



Synthesis and evaluation of anti-microbial and analgesic activity of some (4Z)-3-methyl-1-[(2-oxo-2H-chromen-4-yl) carbonyl]-1H-pyrazole-4, 5-dione 4-[(4-substitutedphenyl) hydrazone]

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Abstract

A series of (4Z)-3-methyl-1-[(2-oxo-2H-chromen-4-yl) carbonyl]-1H-pyrazole-4,5-dione 4-[(4-substitutedphenyl)hydrazone] (**5a-i**) have been synthesized. All the synthesized compounds were characterized on the basis of elemental analysis and spectral data (IR, ¹HNMR). The titled compounds were screened for their anti-inflammatory and analgesic activity. Among the synthesized compounds, compound **5a**, **5c**, **5g** and **5h** exhibited significant anti-microbial activity and compound **5a**, **5b**, **5d**, **5h** and **5i** exhibited significant analgesic activity compared with the standard drug (indomethacin 5mg/kg) at the dose level of 50mg/kg on oral administration.

Keywords: Microbial, pain, coumarin, pyrazolin-5-one.

Introduction

Since resistance of pathogenic bacteria towards available antibiotics is rapidly becoming a major world-wide problem, the design of new compounds to deal with resistant bacteria has become one of the most important areas of antibacterial research today. In addition, primary and opportunistic fungal infections continue to increase rapidly because of the increased number of immune compromised patients. As known, not only biochemical similarity of the human cell and fungi forms a handicap for selective activity, but also the easily gained resistance is the main problem encountered in developing safe and efficient antifungals. Antipyrine [1] (2, 3-dimethyl-1-phenyl-3-pyrazolin-5-one) was the first pyrazolone derivative introduced in the year 1884 for the management of pain, inflammation and fever. Many of these pyrazole derivatives [2-4] such

as phenylbutazone, febrazone, feclobuzone, metobutazone and ramitenazone have found their clinical application as NSAID's. Pyrazolone and their derivatives have attracted the attention of several research groups due to their wide range of pharmacological activities [5-9] which exhibited anti-inflammatory, analgesic, antipyretic, anti-tubercular, anti-bacterial, anticonvulsant, and antidepressant, anticancer. On the other hand coumarins and their derivatives have engrossed substantial attention from organic and medicinal chemists for many years as they belong to a class of compounds with proven utility in medicinal chemistry. Coumarin is an important scaffold since several coumarin derivatives have wide range of biological activities [10-14] such as antimicrobial, antitubercular, anticancer agent etc. As a part of our continued program on the chemistry of pyrazole ring systems, we recently developed a simple and efficient approach to a wide range of such derivatives. These results prompted us to synthesize a series of new pyrazolone derivatives containing coumarin ring that would act as potent anti-microbial and analgesic agent.

Materials and Methods

Experimental

Chemicals

All chemicals used in the study were purchased from SD Fine chemicals and E Merck. Melting points were determined in open glass capillaries using Thomas capillary melting point apparatus. The infrared (IR) spectra were recorded on Jasco FTIR (4100) IR spectrophotometer using the KBr disc technique. The ¹H NMR spectra were recorded on Bruker (NRC-IIsc) Spectrometer. Elemental analysis were performed on Heraeus CHN rapid analyzer. Results of elemental analysis were ± 0.4 % of the theoretical values. Purity of the compounds was checked on TLC plates using silica gel G as stationary phase and iodine vapors as visualizing agent.

Synthesis of Ethyl-2-oxo-2H-chromene-3-carboxylate [15] (1a)

To a cold mixture of salicylaldehyde (0.2M) and diethyl malonate (0.2M), 2 ml of Piperidine was added by rapid stirring. After 20 min the yellow solid separated was filtered off subsequently washed with ethanol and was recrystallized from water: ethanol (2:8), M.p.128°C and yield was 85 %.

Synthesis of 2-oxo-2H-chromene-3-carbohydrazide [12] (2a)

A mixture of Ethyl-2-oxo-2H-chromene-3-carboxylate (0.01M) and hydrazine hydrate 98 % (0.5M) was refluxed for an appropriate time. The reaction mixture was poured into water and the solid which separate filtered off. It was the recrystallized from ethanol to give the title compound, M.p.145°C and yield was 72 %.

Diazotization of substituted aniline [16] (3a-i)

A mixture of different substituted aniline (0.01M) in concentrated HCl (3ml) was cooled to 0-5°C under ice, and cooled sodium nitrite solution (1.5 g in 10ml of water) added to it drop wise during 10 minutes. The reaction mixture was then stirred for 30 minutes.

Synthesis of Ethyl-2(substituted phenyl) hydrazono-3-oxobutyrate [17] (4a-i)

To an ice-cold mixture of the active methylene compound (Ethyl aceto acetate) (0.01M) and sodium acetate (4.10 g ; 0.05M) in ethanol (50 ml), was added drop wise with stirring a solution

of diazonium salt compound (**3a-i**)(0.01M) over 15 minutes. The stirring was continued for 30 minutes and the reaction mixture then left for 2 hours at room temperature. The product was collected and recrystallized from ethanol to give the corresponding hydrazone derivatives (**4a-i**).

Synthesis of (4Z)-3-methyl-1-[(2-oxo-2H-chromen-4-yl) carbonyl]-1H-pyrazole-4,5-dione 4-[(4-substituted phenyl) hydrazone[18] (5a – i)

Ethyl-2(substituted phenyl) hydrazono-3-oxobutyrate (**4a-i**) (0.003 M) dissolved in acetic acid (10-15 ml) was added a solution of 2-oxo-2H-chromene-3-carbohydrazide (**2a**) (0.003 M) in acetic acid (15-20 ml) and the mixture was refluxed for 6 h. The reaction mixture was then allowed to stand overnight. On cooling, the resulting sticky product was dissolved in water ether mixture. From this mixture it was extracted ether (55 %).

Biological Evaluation

Animals

Albino mice of either sex weighing 20-25 g were used for acute toxicity studies and analgesics activity. Healthy male albino adult rats weighing 150-230 g were used for anti-inflammatory screenings. Animals were kept in KMCH College of Pharmacy, Coimbatore, animal are housed individually in polypropylene cages, maintained under standard conditions of alternating 12 h light and dark cycles at a constant temperature (25±2°C and 35-60 % RH). Animals were fed with standard rat pellet and water *ad libitum*.

Acute toxicity

The acute toxicity test was carried out according to an organization for Economic co-operation and development (OECD) guidelines [19] to establish the effective dose of test compounds after obtaining ethical clearance from animal ethics committee of KMCH College of pharmacy, Coimbatore. Albino mice of either sex weighing between 25-30 g were grouped into 9 groups of six animals each, starved for 24 h with water *ad libitum* prior to test. On the drug of experiment animals were administered with different compounds to different groups in an increasing dose of 10, 20, 100, 200, 1000, 1500, 2000, 2500 mg/kg orally. The animals were then observed continuously for 3 h for general behavioral neurological, autonomic profiles and then every 30 min for next 3hr and finally for 24 h or till death.

Analgesic activity

Acetic acid-induced writhing model in mice [20]

Twenty-four hours prior to actual testing a large number of mice (20-25 mg) received intraperitoneally 10 ml/kg of 0.6 % glacial acetic acid. Animals were observed for writhing movements. Only these showing one or other type of writhing movements (positive responders) were chosen for the test on the next day. On the test day the responders received synthesized compounds (**5a-i**) half an hour prior to glacial acetic acid challenge. Synthesized compounds 5a-i was orally administrated at a dose of 50 mg/kg as a suspension in 0.5 Sodium Carboxyl Methyl Cellulose (CMC). Each mouse was then observed for the total number of stretching episodes or writhing for 15minutes following glacial acetic acid injection. Percentage of writhing was calculated using the relation.

$$\text{Inhibition of Writhing (\%)} = 100 \times (1 - b/a).$$

where; a=Mean writhing number of control mice, b=Mean writhing number of treated mice.

Antimicrobial activity - Disk diffusion method [21-23]

The antimicrobial screening of synthesized nine compounds was performed using the disk diffusion method. The selected strains were preserved by periodical sub culturing on agar slant and storing them under frozen condition; for the study fresh 24 hours broth cultures were used. Each bacterial and fungal pure culture was transferred into 100 ml of Muller Hinton nutrient broth and Sabouraud's dextrose broth, respectively. The inoculated broths were incubated at 37°C for 24 hours and 27°C for 72 hours for bacteria and fungus respectively. After incubation, inoculate were standardized to 10⁸ colony-forming units (CFU)/ml for bacteria and 10⁶ CFU/ml for fungus by colony forming unit method. Muller Hinton agar media was prepared by using Beef infusion 300 g, Casein acid hydrolysis 17.5 g, starch 1.5 g, and agar 17 g. Accurately weighed quantities of these ingredients were suspended in 1,000 ml of distilled water. They were boiled to dissolve completely. The pH was adjusted to 7.3 ± 0.2 at 25°C. It was then sterilized by autoclaving at 15 lbs. pressure (121°C for 15 minutes). The prepared Muller Hinton agar medium was transferred into sterile Petri plates; 200 µl of the standardized bacterial inoculums and fungus inoculums were spread on agar medium using sterile cotton swab. The synthesized compounds were dissolved in suitable DMF solvent to a final concentration of 50 µl of drug solution, assuming that each disk absorbed approximately 10 µl of the drug. The drug was impregnated on disk and placed on the inoculated agar medium. Ciprofloxacin and clotrimidazole were used as a standard for the antibacterial and antifungal activity, respectively. All the bacterial Petri plates were kept in an incubator and the fungal Petri plate was kept at room temperature for approximately 18 hours. Then the zones of inhibition were measured.

Results and Discussion**Chemistry**

The synthetic strategies adopted for the synthesis of the intermediate and target compounds are depicted in the Scheme I. Ethyl-2-oxo-2H-chromene-3-carboxylate (**1a**) and 2-oxo-2H-chromene-3-carbohydrazide (**2a**) were synthesized according to the literature procedures. The aryl diazonium salts (**3a-i**) were synthesized by diazotization of different derivatives of anilines using a mixture of sodium nitrite and HCl at 0-5°C. The diazonium salts thus obtained were treated in ethanol in the presence of sodium acetate with calculated amounts of ethyl acetoacetate to afford the corresponding ethyl-2(substituted phenyl) hydrazono-3-oxobutyrate (**4a-i**). 2-oxo-2H-chromene-3-carbohydrazide (**2a**) and ethyl-2(substituted phenyl)hydrazono-3-oxobutyrate (**4a-i**) were dissolved in glacial acetic acid and reflux to form (4*Z*)-3-methyl-1-[(2-oxo-2H-chromen-4-yl)carbonyl]-1*H*-pyrazole-4,5-dione-4-[(4-substitutedphenyl)hydrazone](**5a-i**).

Melting point was uncorrected determined by open capillary tube. Synthesized compounds were characterized by their elemental analysis, IR, ¹H-NMR which is listed in Table I. From the structural investigation, IR spectra showed the stretching frequency range between 1598 and 1640 cm⁻¹, which evinced the presence of imine linkage and also the absence of -NH₂ peak for the synthesized compound derivatives. Dependant substitution of double-bonded nitrogen group of imine C=N could be the reason for the characteristic absorption close to the carbonyl C=O of amide (1630–1680 cm⁻¹) or C=C of alkenes (1600–1680 cm⁻¹) double bond stretching region[24]. ¹H-NMR spectra give a characteristic proton resonance shifts for all the synthesized compound derivatives, which ensured the existence of aromatic, hydrazones, and imines protons.

Scheme-I

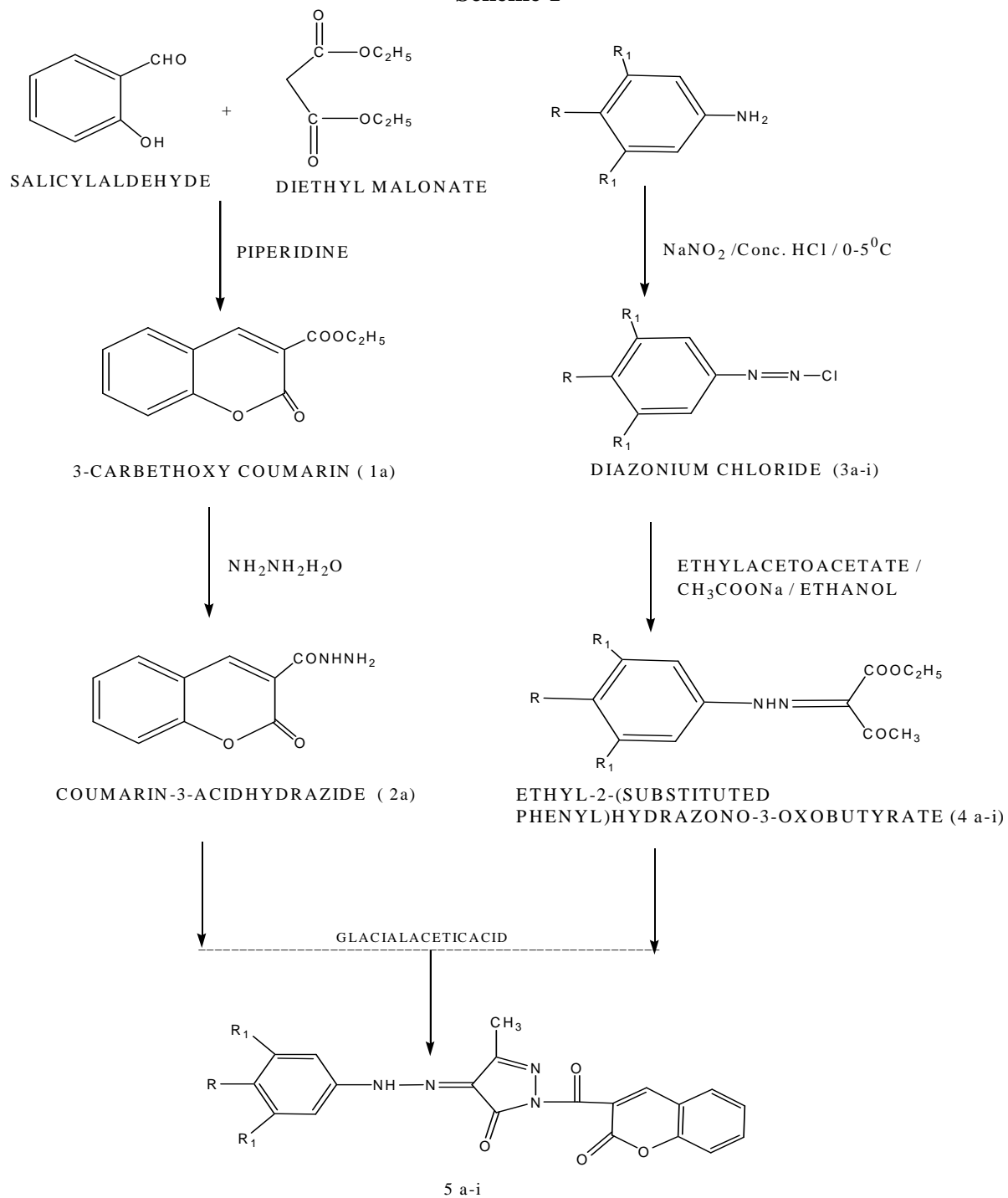


Table- I. Characterization of the synthesized compounds

Compound	Mol wt	m.p. (°C)	Yield (%)	Elemental analysis % calculated/ found				IR (cm ⁻¹)	H ¹ -NMR
				C	H	O	N		
5a	C ₂₀ H ₁₃ ClN ₄ O ₄ 408.79	220	65	58.7 58.2	3.20 3.40	15.68 15.20	13.70 13.60	1685-1680 (C=O), 3445-3210 (NH)1635-1630 (C=N), 3079 (=CH)1717 (C=O, lactone)	δ 7.02(1H, Ar-CH), 6.40 (1H, Ar-CH), 6.90 (1H, NH), 7.65(1H, Ar CH), 0.93(H,CH ₃).
5b	C ₂₀ H ₁₃ BrN ₄ O ₄ 453.25	233	62	52.95 53.02	2.88 2.60	14.11 13.80	12.36 12.40	1672-1678 (C=O), 3480-3209 (NH)1638-1590 (C=N), 3106 (=CH)1708 (C=O, lactone)	δ 7.20(1H, Ar-CH), 6.32 (1H, Ar-CH), 6.86 (1H, NH), 7.57(1H, Ar CH), 0.89(H,CH ₃).
5c	C ₂₀ H ₁₃ FN ₄ O ₄ 392.34	215	60	61.22 60.02	3.33 3.15	16.31 15.02	14.28 13.98	1660-1658 (C=O), 3150-3145 (NH)1638-1636 (C=N), 3086 (=CH)1719 (C=O, lactone)	δ 6.85(1H, Ar-CH), 6.55 (1H, Ar-CH), 6.7 (1H, NH), 7.59(1H, Ar CH), 0.94(H,CH ₃).
5d	C ₂₁ H ₁₆ N ₄ O ₄ 388.37	240	68	64.88 62.80	4.15 4.02	16.47 16.60	14.42 14.02	1668-1665 (C=O), 3145-3143 (NH)1636-1634 (C=N), 3076 (=CH)1724 (C=O, lactone)	δ 6.84(1H, Ar-CH), 6.50 (1H, Ar-CH), 7.10 (1H, NH), 7.35(1H, Ar- CH), 0.92(H,CH ₃), 2.45 (3H,Ar-CH ₃).
5e	C ₂₁ H ₁₆ N ₄ O ₅ 404.37	225	72	62.37 58.26	3.98 3.60	19.78 18.90	13.85 13.60	1655-1656 (C=O), 3442-3158 (NH)1640-1638 (C=N), 3106 (=CH)1720 (C=O, lactone)	δ 6.54(1H, Ar-CH), 6.32(1H, Ar-CH), 6.79 (1H, NH), 7.65(1H, Ar CH), 0.95(H,CH ₃), 3.76(3H,ArCH ₃).
5f	C ₂₀ H ₁₃ N ₅ O ₆ 419.34	240	62	57.28 58.02	3.12 3.06	22.89 22.92	16.70 16.75	1682-1670 (C=O), 3450-3190 (NH)1640-1625 (C=N), 3065 (=CH)1712 (C=O, lactone)	δ 8.12(H, Ar-CH), 6.85(1H, Ar-CH), 7.1 (1H, NH), 7.35(1H, Ar CH), 0.91(H,CH ₃).
5g	C ₂₀ H ₁₂ N ₆ O ₈ 464.34	224	65	51.73 50.92	2.79 2.08	27.56 27.90	18.09 17.90	1695-1660 (C=O), 3340-3210 (NH)1658-1620 (C=N), 3105 (=CH)1718 (C=O, lactone)	δ 8.32(1H, Ar-CH), 7.40(1H, Ar-CH), 9.92 (1H, NH), 7.32(1H, Ar CH),8.7(H,CH ₃).
5h	C ₂₀ H ₁₂ Cl ₂ N ₄ O ₄ 443.24	232	60	54.19 53.90	2.72 2.60	14.43 13.21	12.64 12.06	1650-1608 (C=O), 3250-3100 (NH)1680-1650 (C=N), 3112 (=CH)1725 (C=O, lactone)	δ 7.12(1H, Ar-CH), 6.28(1H, Ar-CH), 7.02 (1H, NH), 7.25(1H, Ar CH), 0.86(H,CH ₃).

5i	C ₂₀ H ₁₂ Br ₂ N ₄ O ₄	230	64	45.14	2.27	12.10	10.52	1705-1650 (C=O),	δ 7.02(1H, Ar-CH),
	532.13			45.06	2.30	11.70	10.20	3150-3042 (NH)1638-1635 (C=N), 3108 (=CH)1714 (C=O, lactone)	6.40(1H, Ar-CH), 6.90 (1H, NH), 7.65(1H, Ar CH), 0.87(H,CH ₃).

Acute toxicity

The compound showed a high safety margin when screened at graded doses 10 mg/kg-2.5mg/kg for their acute lethal doses (ALD₅₀). The (LD₅₀) values were found to be more than 2.5 mg/kg.

Analgesic activity

Table-II reveals the analgesic activity of series of (4Z)-3-methyl-1-[(2-oxo-2H-chromen-4-yl) carbonyl]-1H-pyrazole-4,5-dione 4-[(4-substitutedphenyl) hydrazone] (**5a – i**) at a dose of 50 mg/kg body weight by acetic acid induced writhing method. Abdominal constriction response induced by acetic acid is a sensitive procedure to establish efficacy of peripherally acting analgesic. The synthesized compounds **5a**, **5b**, **5d**, **5h** and **5i** show significant analgesic activity after 3 h acetic acid induced writhing model and all other synthesized compounds shows moderated to good analgesic activity after 3 h at 50 mg/kg when orally administrated in mice compared to standard drug indomethacin 5 mg/kg dose level. The preliminary in vivo analgesic activities of synthesized compounds evidenced that the presence of 4-chloro, 4-Bromo, 3, 4-dichloro, 3, 4-dibromo and 4-methyl group in the aromatic ring of 4-position of the pyrazolone nucleus gave rise to an increased analgesic activities.

Table-II. Analgesic activity of synthesized compounds by acetic acid induced writhing method

Compound ^a	No. of wriths in 15 min (mean ± SEM)	% Protection	ED ₅₀ mg/kg
Control	66.9 ± 0.43	-	
5a	25.5 ± 0.04**	61.8	23.6
5b	27.2 ± 0.24**	59.3	20.0
5c	39.4 ± 0.43*	41.1	-
5d	30.2 ± 0.23**	54.8	10.5
5e	40.0 ± 0.21*	40.2	-
5f	41.9 ± 0.23	37.3	-
5g	44.6 ± 0.32	33.3	-
5h	31.2 ± 0.22**	53.3	30.6
5i	33.9 ± 0.26**	50.0	23.8
Indomethacin	20.4 ± 0.43***	69.5	4.0

*The result are expressed as mean ± SEM (n=6). Significance was calculated by using one-way ANOVA with Dunnet's t-test. The different in result was considered significant when p < 0.05, *p < 0.05 vs control at 50 mg/kg, **p < 0.001 vs control at 50 mg/kg; ***p < 0.001 vs control at 50 mg/kg, ^a Dose test compounds (50 mg/kg), indomethacin (5 mg/kg).

Antimicrobial activity

In the present study four gram-positive, four-gram negative, and three fungus were selected. The gram positive strains were *Bacillus lentus* (NCIM 2018), *Bacillus cereus* (NCIM 2018), *Micrococcus luteus* (NCIM 2155), *Staphylococcus albus* (NCIM 2178); gram negative strains were *Escherichia coli* (NCIM 2065), *Klebsiella aerogenes* (NCIM 2075), *Salmonella paratyphi* (NCIM 2075), *Proteus vulgaris* (NCIM 2239), and fungus *Candida albicans* (NCIM 0707), *Aspergillus niger* (NCIM 3010), *Asperillus orzane* (NCIM 3015). The strain was confirmed for its purity and identity by the gram-staining method and it was further characterized by chemical reaction. The antimicrobial screening of all the compounds showed a significant zone of inhibition against both gram-positive and gram-negative bacteria compared to the standard ciprofloxacin. Similarly, the zone of inhibition on the fungal strain showed a stronger activity as the standard clotrimidazole. New synthesized compound exhibits stronger inhibition on gram-negative *Klebsiella aerogenes* compared with other bacterial strains. On the other hand, *Candida albicans* zone was highly inhibited by new synthesized compound, which proves the efficiency of antifungal activity than antibacterial activity. Discussing the antimicrobial activity against individual compound, it was clear that mono; di halogen substituted compounds have significant inhibitions both gram positive, gram negative bacterial strain as well as fungal strain. It was found that the *Klebsiella aerogenes* and *Candida albicans* were highly susceptible to killing by the synthesized compound. The antimicrobial activity which is listed in Table-III

Table-III Diameter of zone of inhibition by individual compounds against gram-positive, gram-negative bacteria and fungus

Zone of inhibition in mm		Compounds									
Organism	STD ^a	5a	5b	5c	5d	5e	5f	5g	5h	5i	Solvent ^b
Gram+ve bacteria											
<i>Bacillus lentus</i>	16	14	15	12	09	08	10	13	13	15	05
<i>Bacillus cereus</i>	18	15	14	14	10	07	11	15	14	16	04
<i>Micrococcus luteus</i>	16	13	14	13	09	08	11	14	12	15	06
<i>Staphylococcus albus</i>	17	16	13	13	08	09	12	15	13	14	05
Gram-ve bacteria											
<i>Escherichia coli</i>	18	18	15	09	11	09	13	11	14	15	06
<i>Klebsiella aerogenes</i>	15	16	15	14	11	12	11	13	16	17	04
<i>Proteus vulgaris</i>	16	15	13	13	09	10	09	11	13	13	05
<i>Salmonella paratyphi</i>	17	14	15	13	13	09	11	13	14	15	05
Fungus											
<i>Candida albicans</i>	14	15	14	13	13	12	12	14	16	15	05
<i>Aspergillus niger</i>	16	14	12	12	11	12	13	13	15	14	04
<i>Aspergillus orzane</i>	17	13	15	12	12	14	12	15	16	18	06

^a Standard ciprofloxacin for bacteria, clotrimazole for fungal

^b DMF

Conclusion

In the present paper, we report the synthesis, spectral studies, evaluation of antimicrobial and analgesic activity of (4Z)-3-methyl-1-[(2-oxo-2H-chromen-4-yl) carbonyl]-1H-pyrazole-4,5-

dione 4-[(4-substitutedphenyl)hydrazone] (5a – i). Thus these compounds constitute an interesting template for the evaluation of new anti microbial and analgesic inhibitors. In conclusion, we reported herein a simple and convenient route for the synthesis of some new heterocyclic based on pyrazolinone for antimicrobial evaluation and analgesic activity.

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