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Synthesis and biological evaluation of pyridopyrimidinone derivatives containing thiophene ring as potential anti-inflammatory and antimicrobial agents

Gollapalli Naga Raju*[1a], Kondeti T Naveen[2b], K. Chandana[2a] and Rama Rao Nadendla[1a]

[1]Department of Pharmaceutical Analysis
[2]Department of Pharmacy Practice
[a]Chalapathi Institute of Pharmaceutical Sciences, Guntur, India.
[b]Rao's college of Pharmacy, Nellore, India

ABSTRACT

Thiophene is an important class of heterocyclic compound, has been shown to exhibit diverse biological and pharmacological activities such as anti-cancer, antioxidant, anti-inflammatory, antimicrobial, etc. In this study, a series of novel 2-Methyl-5-thiophen-7-(aryl-2-yl) pyrido[2,3-d]pyrimidin-4(3H)-one derivatives have been synthesized. All the synthesized compounds have been characterized by using elemental analysis, FT-IR, ^{1}H NMR, ^{13}C NMR spectroscopy and further supported by mass spectroscopy. Purity of all the compounds has been checked on thin layer chromatographic plate and HPLC technique. All the synthesized compounds were tested for their antibacterial and antifungal activity (MIC) in vitro by broth dilution method with two Gram-positive bacteria, two Gram-negative bacteria and two fungal strains. The biological activities of the synthesized compounds have been compared with standard drugs Ampicillin and Greseofulvin..The compounds exhibited significant antibacterial and moderate antifungal activities. These compounds can be further exploited to get the potent lead compounds. The detailed synthesis and the antimicrobial screening of the new compounds are reported.

Keywords: Pyridopyrimidinone, Thiophene, Antibacterial activity, Antifungal activity.

INTRODUCTION

It has been observed that spite of mounting problems of resistance to antimicrobial agents, the number of innovative antibiotics being brought to the pharmaceutical market have been reduced drastically in recent times.Here is shortage of novel antibiotics and appearance of multi-drug conflicting microbes being some of the major challenges for drug proposal and expansion of narrative antimicrobial agents.

Heterocyclic compounds containing sulphur have considerably a lot of attention due to wide application of pharmacological activity. Substituted thiophenes and their biheterocycles have received considerable attention during last two decades as they are endowed with wide range of therapeutic properties. A number of thiophene derivatives and Schiff bases have been reported to possess significant and diverse biological activities such as antibacterial [1, 2], antifungal [3, 4], antimicrobial [5, 6], anti-inflammatory [7,8],antioxidant [9, 10], antitumor [11, 12], Anti-leishmanial [13], antidepressant [14], antidiabetic [15], and local anesthetic [16] activities. Thiophene can be fused with various heterocyclic nuclei giving rise to newer compounds having enhanced biological activities.

In continuation to these efforts and with an objective to develop novel and potent therapeutic agents of synthetic origin, it was decided to synthesize certain 7-(Thiophen-2-yl)pyrido[2,3-d]pyrimidin-4(3H)-one derivatives and evaluate them for their antimicrobial potential.

MATERIALS AND METHODS

Melting points were determined in open capillary tubes and are uncorrected. Formation of the compounds was checked by TLC on silica gel-G plates of 0.5 mm thickness and spots were located by iodine and UV light. All compounds were purified by recrystallization with suitable organic solvents. IR spectra were recorded on Brooker-ALPHA FT-IR instrument using KBr pellet method. Mass spectra were recorded on Shimadzu GC-MS-QP-2010 model using direct inlet probe technique. Purity of the synthesized compounds was checked by HPLC Agilent. The results are in agreements with the structures assigned. Elemental analysis of the all the synthesized compounds was carried out on Euro EA 3000 elemental analyzer and the results are in agreements with the structures assigned.

Preparation of 3-Phenyl-1-(thiophen-2-yl)prop-2-en-1-one.
These were prepared by condensation of 2-acetylthiophene and substituted aryl aldehyde in the presence of sodium hydroxide.

General procedure for the preparation of 2-Methyl-5-aryl-7-(thiophen-2-yl)pyrido[2,3-d]pyrimidin-4(3*H*)-one: A mixture of an appropriate2-methyl-4-aryl-6-(thiophen-2-yl)pyridine-3-carbonitrile (0.01 mol) and acetic acid (20 ml) was stirred under reflux for 12-14 hour in the presence of catalytic amount of concentrated H_2SO_4. The reaction mixture was allowed to cool to room temperature and was poured into ice cold water. The solid thus formed was collected by filtration, washed with chloroform (20 ml) and the resulting crude product was crystallized from methanol to give the analytical pure compound. The physical constants of the product are recorded in Table-1.

5-(4-Fluorophenyl)-2-methyl-7-(thiophen-2-yl)pyrido[2,3-*d*]pyrimidin-4(3*H*)-one(TP-1): IR (KBr): 3364, 3060, 3045, 2891, 1693, 1585, 1558, 1545, 1463,1075, 851 cm^{-1}; MS: *m/z* = 338 $[M+1]^+$; Anal. Calcd for $C_{18}H_{12}FN_3OS$: C, 64.08; H, 3.59;N, 12.46. Found: C, 63.18; H, 3.41; N, 12.26%.

5-(3-Chlorophenyl)-2-methyl-7-(thiophen-2-yl)pyrido[2,3-*d*]pyrimidin-4(3*H*)-one (TP-2):IR (KBr): 3468, 3051, 3008, 2960, 1698, 1618, 1604, 1578, 1471,1078, 770 cm^{-1}; MS: *m/z* = 355 $[M]^+$; Anal. Calcd for $C_{18}H_{12}ClN_3OS$: C, 61.10; H, 3.42;N, 11.88. Found: C, 60.58; H, 3.17; N, 11.53%.

2-Methyl-5-(4-methylphenyl)-7-(thiophen-2-yl)pyrido[2,3-*d*]pyrimidin-4(3*H*)-one(TP-3): IR (KBr): 3504, 3145, 2978, 2861, 1712, 1645, 1618, 1563, 1545,1063, 841 cm^{-1}; MS: *m/z* = 333 $[M]^+$; Anal. Calcd for $C_{19}H_{15}N_3OS$: C, 68.45; H, 4.53; N,12.60. Found: C, 67.57; H, 4.30; N, 12.49%.

2-Methyl-5-phenyl-7-(thiophen-2-yl)pyrido[2,3-*d*]pyrimidin-4(3*H*)-one (TP-4):IR (KBr): 3341, 3029, 2983, 2866, 1709, 1595, 1583, 1568, 1478, 1018 cm^{-1};MS: *m/z* = 319 $[M]^+$; Anal. Calcd for $C_{18}H_{13}N_3OS$: C, 67.69; H, 4.10; N, 13.16. Found: C,67.09; H, 3.97; N, 13.05%.

5-(4-Chlorophenyl)-2-methyl-7-(thiophen-2-yl)pyrido[2,3-*d*]pyrimidin-4(3*H*)-one(TP-5): IR (KBr): 3309, 3151, 2998, 2905, 1691, 1683, 1578, 1543, 1518,1023, 854 cm^{-1}; MS: *m/z* = 354 $[M+1]^+$; Anal. Calcd for $C_{18}H_{12}ClN_3OS$: C, 61.10; H,3.42; N, 11.88. Found: C, 60.48; H, 3.21; N, 11.49%.

2-Methyl-5-(2-nitrophenyl)-7-(thiophen-2-yl)pyrido[2,3-*d*]pyrimidin-4(3*H*)-one (TP-6):IR (KBr): 3290, 3043, 3023, 2818, 1890, 1609, 1590, 1579, 1449, 1065,720 cm^{-1}; MS: *m/z* = 364 $[M]^+$; Anal. Calcd for $C_{18}H_{12}N_4O_3S$: C, 59.33; H, 3.32; N,15.38. Found: C, 58.86; H, 3.18; N, 15.09%.

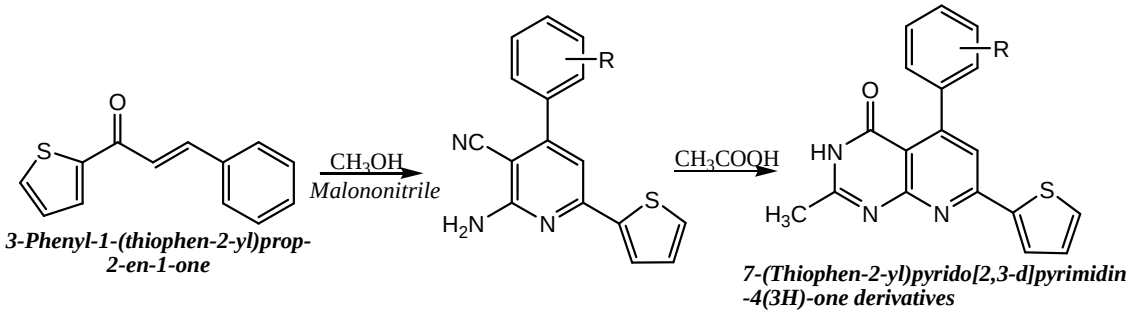
2-Methyl-5-(4-nitrophenyl)-7-(thiophen-2-yl)pyrido[2,3-*d*]pyrimidin-4(3*H*)-one (TP-7): IR (KBr): 3351, 3031, 3015, 2957, 1876, 1602, 1589, 1578, 1479, 1043,710 cm^{-1}; MS: *m/z* = 364 $[M]^+$; Anal. Calcd for $C_{18}H_{12}N_4O_3S$: C, 59.33; H, 3.32; N,15.38. Found: C, 56.87; H, 3.15; N, 15.27%.

5-(4-Aminophenyl)-2-methyl-7-(thiophen-2-yl)pyrido[2,3-*d*]pyrimidin-4(3*H*)-one(TP-8):IR (KBr): 3481, 3147, 2969, 2884, 1707, 1578, 1563, 1533, 1469,1021, 844 cm^{-1}; MS: *m/z* = 334 $[M]^+$; Anal. Calcd for $C_{18}H_{14}N_4OS$: C, 64.65; H, 4.22; N,16.75. Found: C, 64.01; H, 4.16; N, 16.58%.

5-(4-Bromophenyl)-2-methyl-7-(thiophen-2-yl)pyrido[2,3-*d*]pyrimidin-4(3*H*)-one(TP-9): IR (KBr): 3501, 3170, 3046, 2930, 1681, 1619, 1615, 1578, 1503,1056, 866 cm^{-1}; MS: *m/z* = 399 $[M+1]^+$; Anal. Calcd for $C_{18}H_{12}BrN_3OS$: C, 54.28; H,3.04; N, 10.55. Found: C, 53.81; H, 2.91; N, 10.41%.

5-(3-Methoxyphenyl)-2-methyl-7-(thiophen-2-yl)pyrido[2,3-*d*]pyrimidin-4(3*H*)-one(TP-10):IR (KBr): 3349, 3093, 29636, 2847, 1680, 1620, 1595, 1583, 1493,1037, 767 cm^{-1}; MS: *m/z* = 349 $[M]^+$; Anal. Calcd for $C_{19}H_{15}N_3O_2S$: C, 65.31; H, 4.33; N,12.03. Found: C, 64.72; H, 4.23; N, 11.87%.

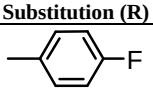
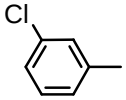
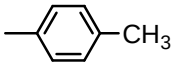
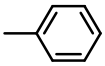
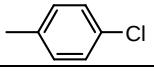
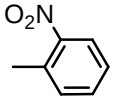
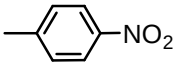
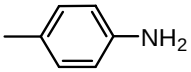
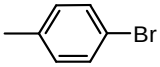
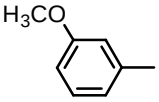
Scheme 1: Synthesis of 7-(thiophen-2-yl)pyrido[2,3-d]pyrimidin-4(3H)-one derivatives



3-Phenyl-1-(thiophen-2-yl)prop-2-en-1-one

7-(Thiophen-2-yl)pyrido[2,3-d]pyrimidin-4(3H)-one derivatives

Table-1: Physical constants of 5-(Thiophen-2-yl)pyrido[2,3-d]pyrimidin-4(3H)-one derivatives (TP-1 to TP-10)

Compd	Substitution (R)	M.F	M.W	M.P (^{0}C)
TP-1		$C_{18}H_{12}FN_3OS$	337.37	201-203
TP-2		$C_{18}H_{12}ClN_3OS$	353.82	256-258
TP-3		$C_{19}H_{15}N_3OS$	333.40	294-296
TP-4		$C_{18}H_{13}N_3OS$	319.38	216-217
TP-5		$C_{18}H_{12}ClN_3OS$	353.82	176-177
TP-6		$C_{18}H_{12}N_4O_3S$	364.37	181-183
TP-7		$C_{18}H_{12}N_4O_3S$	364.37	166-167
TP-8		$C_{18}H_{14}N_4OS$	334.39	210-211
TP-9		$C_{18}H_{12}BrN_3OS$	398.27	248-250
TP-10		$C_{19}H_{15}N_3O_2S$	349.40	229-230

BIOLOGICAL EVALUATION[17]**:**

Preparation of Culture Media: Nutrient broth was used as growth medium for bacteria and Saubouraud dextrose broth for fungi. Nutrient broth was prepared by dissolving 13gm of dehydrated powder (HI-media) in 100ml of distilled water. Saubouraud dextrose broth was prepared by dissolving 4gm of dextrose and 1gm of peptone in 100ml of distilled water. The media were sterilized by autoclaving at 15lbs pressure for 20 minutes.

Preparation of Stock Culture: Stock cultures were obtained by aseptically transferring a loopful of test organisms to 100ml of sterile broth and incubated for 24 hours at 37^0C.

Standardization of Stock Culture: Stock cultures were placed in the incubator (37^0C for bacteria and 24^0C for fungi) and shaken well. One ml of stock cultures was aseptically transferred to 9 ml of sterile water containing 0.05% tween 80. This was mixed with using a cyclomixer and serially diluted from 10^{-1} to 10^{-10}. From each dilution, 0.2ml was taken and spread on sterile nutrient agar plates for bacteria and Sabouraud dextrose agar plates for fungi, which were incubated for 18 hours. After incubation, the numbers of colonies in the plate were counted. The number of colonies for a plate that was formed from the maximum dilute tube was noted. The number of microorganisms in stock were then calculated and expressed as colony forming units per ml (cfu/ml). By back calculation the stock culture was found to contain 15×10^8cfu/ml.

Preparation of Working Stock Culture: Stock culture (0.1ml) was diluted with nutrient broth (100ml) and Sabouraud dextrose broth (100ml) respectively to obtain 10^5cfu/ml. This was then used for further *in vitro* screening.

Preparation of Drug Dilutions: Solutions of the title compounds in DMSO (1mg/ml) were prepared and used for screening their antimicrobial activity.

Antimicrobial Screening: Synthesized compounds were subjected to antimicrobial screening by estimating the minimum inhibitory concentration (MIC) by adopting serial dilution technique. Test was carried out on four bacterial strains, namely *Staphylococcus aureus*(MTCC 96), *Staphylococcuspyogenus, Pseudomonas aeruginosa*(MTCC 1688),*Escherichia coli* (MTCC 443) and two fungal strains, namely *Candida albicans*(MTCC 227) and *Aspergilla niger* (MTCC 282).

Determination of MIC: The study involved a series of six assay tubes for each title compound against each microorganism. The entire test was done in duplicate. To the first assay tube, 1.8ml of seeded broth and 0.2ml of title compound (1mg/ml) was added and mixed thoroughly and the two fold serial dilution was done up to the sixth tube containing 1 ml of seeded broth. The additions of the drug solution and serial dilution were done under strict aseptic conditions. Solvent control, negative control (growth control) and drug control were maintained during the experiment. The assay tubes were incubated at 37^0C and 25^0C respectively for 24 hours for bacteria and fungi. The lowest concentration, which apparently caused complete inhibition of growth of microorganisms, was considered as the minimum inhibitory concentration (MIC). The results obtained from antimicrobial susceptibility testing are depicted in Table 2.

Table-2: Antimicrobial activity of 7-(Thiophen-2-yl)pyrido[2,3-d]pyrimidin-4(3H)-one derivatives (TP-1 to TP-10)

Compound	Minimal Inhibitory Concentration (µg/ml)					
	Antibacterial Activity				Antifungal activity	
	S.aureus	***S.pyogenus***	***E.coli***	***P.aeruginosa***	***C.albicans***	***A.niger***
TP-1	200	500	62.5	100	500	200
TP-2	250	100	100	500	1000	200
TP-3	100	200	500	250	500	250
TP-4	500	125	500	200	250	500
TP-5	250	100	100	200	200	500
TP-6	200	250	200	250	1000	1000
TP-7	500	250	250	200	500	200
TP-8	500	200	100	250	200	250
TP-9	200	250	250	200	250	500
TP-10	200	100	500	100	250	500
Ampicillin	250	100	100	100	NT	NT
Greseofulvin	NT	NT	NT	NT	500	100

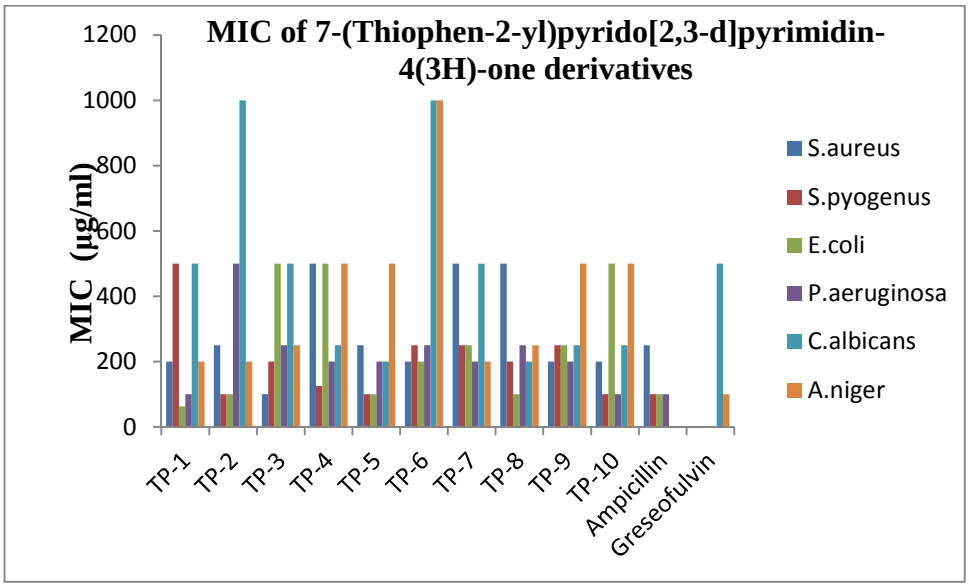


Figure 1: Antimicrobial activity of 7-(Thiophen-2-yl)pyrido[2,3-d]pyrimidin-4(3H)-one derivatives

ANTI-INFLAMMATORY ACTIVITY: Carrageenan-induced rat paw edema method employing Zeitlin's apparatus was used to determine the anti-inflammatory activity of the newly synthesized Pyridopyrimidinone derivatives containing thiophene ring.

Materials: Carrageenan required for inducing the inflammation was obtained from Himedia (Mumbai) whereas sodium CMC was of Merck grade and the required saline (Core Health Care) was purchased from a local supplier. Aceclofenac used as standard was supplied as a gift sample by Jagsonpal, New Delhi.

Preparation of sodium CMC suspension: 1gm of sodium CMC was triturated in 100 ml of distilled water to give the required stock suspension of sodium CMC. This stock suspension was used for suspending all the test compounds as well as the standard drug.

Experimental procedure: Albino rats of either sex, weighing between 150-200 gm, supplied by M/S Ghosh Enterprises, Kolkata were divided into twenty seven groups of six animals each. All these groups were kept for fasting overnight and only allowed water *adlibitum*.

0.05 ml of 1% carrageenan suspension was slowly injected subcutaneously into the subplantar region of the left hind paw to produce inflammation in all the groups. Groups III to XXVII were treated with 7-(Thiophen-2-yl)pyrido[2,3-d]pyrimidin-4(3H)-one derivatives (TP-1 to TP-10) (10 mg/kg) after carrageenan administration and the time gap is at an interval of 0.5, 1, 2, 3, 4 and 6 h. Group I used as carrageenan treated control was given only 1% sodium CMC suspension (1 mL/kg) whereas group II received aceclofenac (2 mg/kg). All these doses were administered orally and the induced paw edema in each group was measured to assess the anti-inflammatory activity.

Measurement of paw thickness: Before carrageenan injection, the thickness of both the paws of each rat was measured using Zeitlin's constant load lever method. The paws thickness was also measured in a similar way after carrageenan injection at time intervals 0, 0.5, 1, 2, 3, 4 and 6 h. The dose selection for the compound in the preliminary screening is usually 5 times the dose of the standard drug aceclofenac, which was used at a dose of 2 mg/kg.

The percent increase at each time interval was determined by using the formula: Yt-Yo / Yo×100
Yt = Paw thickness at time t hours (after injection),
Yo = Paw thickness at time 0 hours (before injection)

The percent inhibition of paw oedema thickness was calculated by using the formula:

Percentage inhibition = [1- Yt/Yc] × 100

Where Yt= Average increase in paw thickness in groups tested with Pyridopyrimidinone derivatives containing thiophene ring and the standard.

Yc= Average increase in paw thickness in control

The results of anti-inflammatory activity of aceclofenac and thePyridopyrimidinone derivatives containing thiophene ring compounds tested are shown inTable 3.

Table 3: Percentage inhibition in paw thickness at various time intervals

Compound	% inhibition in paw thickness at various time intervals					
	0.5 Hr	**1 Hr**	**2 Hr**	**3 Hr**	**4 Hr**	**6 Hr**
TP-1	19 ± 1	22 ± 1	57 ± 1**	67 ± 1	96 ± 1	97 ± 2
TP-2	16 ± 1	* 19 ± 2	52 ± 1	62 ± 1**	90 ± 1	92 ± 1
TP-3	10 ± 1	14 ± 2	46 ± 1	55 ± 1*	84 ± 1**	86 ± 2*
TP-4	03 ± 1	07 ± 1**	39 ± 2	48 ± 1	73 ± 1	74 ± 2
TP-5	18 ± 1	20 ± 1	54 ± 1*	64 ± 1	93 ± 1	94 ± 2
TP-6	16 ± 1	18 ± 2	52 ± 2	61 ± 1	89 ± 1	90 ± 1*
TP-7	16 ± 1	18 ± 1	52 ± 1**	62 ± 1	89 ± 1	90 ± 1
TP-8	06 ± 1	11 ± 1	42 ± 1	52 ± 1	80 ± 1	82 ± 2
TP-9	18 ± 1	20 ± 1	55 ± 1	65 ± 1*	94 ± 1	95 ± 2
TP-10	10 ± 1*	14 ± 2	47 ± 1	56 ± 2	84 ± 2	87 ± 1
Standard (Aceclofenac)	22 ± 1	25 ± 1	59 ± 1	68 ± 1	98 ± 2	99 ± 1

Values are expressed as mean ±(n=6)
P<0.05, P**<0.01 compared to control, Student t-test (Unpaired)*
Value for the control group in all the cases is zero

RESULTS AND DISCUSSION

Condensation of 2-acetylthiophene and substituted aryl aldehyde in the presence of sodium hydroxide forms the 3-Phenyl-1-(thiophen-2-yl)prop-2-en-1-one. A mixture of 2-methyl-4-aryl-6-(thiophen-2-yl)pyridine-3-carbonitrile and acetic acid was stirred under reflux for 12-14 hour in the presence of catalytic amount of concentrated H_2SO_4 forms the 7-(Thiophen-2-yl)pyrido[2,3-d]pyrimidin-4(3H)-one derivatives. All the synthesized compounds were subjected to antimicrobial screening by estimating the minimum inhibitory concentration (MIC) by adopting serial dilution technique.

The data recorded in Table 2 indicated that compound**TP-3** is more potent towards *Staphylococcus aureus* The compounds **TP-1, TP-2, TP-5, TP-6, TP-9** and **TP-10** are moderately potent towards the *Staphylococcus aureus.* Compounds **TP-2, TP-4, TP-5** and **TP-10**aremoderately potent towards the *Streptococcus pyogenes.* Compound **TP-1** is more potent towards the *Escherichia coli* and compounds **TP-2, TP-5** and **TP-10** were moderately potent towards the *Escherichia coli.*Compounds **TP-1** and **TP-10**aremoderately potent towards the *Pseudomonas aeruginosa.*All these compounds are compared with the standard reference (Ampicillin) for their antibacterial activities. The compounds **TP-4, TP-5, TP-8, TP-9** and **TP-10** are more potent towards the *Candida albicans.*The compounds **TP-1, TP-3** and **TP-7** are moderately potent towards the *Candida albicans.* All these compounds are compared with the standard reference (Greseofulvin) for their antifungal activities.

The anti-inflammatory activity of the newly synthesized 7-(Thiophen-2-yl)pyrido[2,3-d]pyrimidin-4(3H)-one derivatives**(TP-1 To TP-10)** has been evaluated by using carrageenan-induced rat paw edema method. The results of the evaluation have been viewed by taking aceclofenac as the standard drug.

The results of anti-inflammatory activity revealed that the compounds **TP-1 To TP-10**exhibited moderate to considerable activity when compared with reference standard aceclofenac, but not at an identical dose level as the standard drug was tested at 2 mg/kg, whereas the chalcones were tested at a dose of 10 mg/kg. Pyridopyrimidinone derivatives containing thiophene ring compounds**TP-2**, **TP-5**, **TP-1**, and **TP-9**having the electron withdrawing groups like the halogens showed maximum activity and this is consistent with the literature reports that such groups enhance the lipophilic properties of the molecule. Other compounds tested in this present study also showed some degree of anti-inflammatory activity. Some of these compounds were substituted with electron releasing substituents on the aromatic ring at different positions.

CONCLUSION

In this study, certain 7-(Thiophen-2-yl)pyrido[2,3-d]pyrimidin-4(3H)-one derivatives were synthesized and evaluated for theirantimicrobial& anti-inflammatory activities. Results revealed that the compounds exhibited significant *in-vitro* activity. All the synthesized compounds are more potent to moderate antimicrobial activities against the test organisms. Pyridopyrimidinone derivatives containing thiophene ring compoundshaving the electron withdrawing groups like the halogens showed maximum activity. Other compounds tested in this present study also showed some degree of anti-inflammatory activity.The study would be a fruitful matrix for the development of 7-(Thiophen-2-yl)pyrido[2,3-d]pyrimidin-4(3H)-one derivatives for further bio-evaluation.

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