(IJPS) 2023, Vol. No. 16, Issue 1, Jul-Dec **e-ISSN: 2455-6270 p-ISSN: 2455-7455**

Synthesis, Molecular Docking studies, Anticancer and Anti-Inflammatory Activity of Tetrazole Linked Isoxazolo[5,4-*B*]Pyridines[1]

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DOI: 10.37648/ijps.v16i01.009

Received: 05 August 2023; Accepted: 27 September 2023; Published: 11 October 2023

Abstract

Objective: A series of tetrazole linked isoxazolo[5,4-*b*]pyridines were synthesized, and their anticancer, anti-inflammatory activity, and molecular docking studies were evaluated. **Methods:** The one-pot reaction of 5-amino-3-methylisoxazole **1**, substituted aromatic aldehyde **2**, and benzoyl acetonitrile **3** in presence of $FeCl_3$ on basic alumina (1:2 equiv.) furnished 3-methyl-4-aryl-6-phenylisoxazolo[5,4-*b*]pyridine-5-carbonitriles **4**. Compounds **4** on reaction with benzyl azides in presence of saturated copper sulfate solution and Cu-turnings in ethanol afforded the title compounds *viz.,* 5-(2-benzyl-2*H*-tetrazol-5-yl)-3-methyl-4-aryl-6-phenylisoxazolo[5,4-*b*]pyridines **5a-l**. The anticancer activity of the synthesized compounds was determined using MTT assay for *in vitro* studies, liquid tumor model for *in vivo* activity. The anti-inflammatory activity and molecular docking studies has been carried out. **Results and Discussion:** The structures of newly synthesized derivatives were confirmed by IR, ^1H, ^{13}C NMR and mass spectrometry. In anticancer activity as well as in anti-inflammatory studies, among all the derivatives **5a-l**, **5c**, **5d** exhibited potent activity against the HeLa, EAC and MCF-7 cell lines. They also demonstrated significant *in vitro* cyclooxygenase inhibition (COX-1, COX-2) during anti-inflammatory activity. These results very well supported by molecular docking analysis. **Conclusions:** A new series of tetrazole linked isoxazolo[5,4-*b*]pyridines were developed and investigated for *in vitro* and *in vivo* anticancer activity, anti-inflammatory activity, and molecular docking studies. Compounds **5c**, **5d** showed promising results in both anticancer and anti-inflammatory assays, and the results are very well supported by molecular docking analysis. These scaffolds, with further structural modification, hold potential candidates for cancer and inflammatory therapeutics.

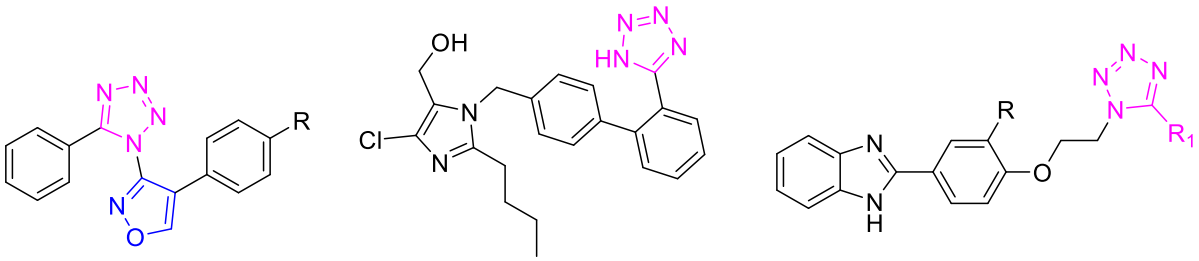
Keywords: *Three-component synthesis; tetrazole linked isoxazolo[5,4-b]pyridines; anticancer agents; anti-inflammatory activity; molecular docking studies; cyclooxygenase inhibition studies.*

Introduction

Cancer is a major health problem worldwide and the major cause of human mortality in the world as recognized by World Health Organization (WHO) [1]. The identification of novel structures for effective treatment of cancer is still a major challenge to medicinal chemists [2-4]. Over the time, important milestones have been achieved in the development of various lung, breast, stomach and colorectal cancer static drugs for antitumor chemotherapy. Although chemotherapy is one of the primary treatment therapies currently, it has certain limitations such as serious toxic effects and drug resistance [5]. Therefore, there is an urgent need to search for more efficient, better tolerated anticancer drugs [6]. Epidermal Growth Factor Receptor (EGFR), trans-membrane tyrosine kinase inhibitor is expected to have

[1] *How to cite the article:* Thirupathaiah K., Goud G.S., Gopal N.V; Jul-Dec 2023; Synthesis, Molecular Docking studies, Anticancer and Anti-Inflammatory Activity of Tetrazole Linked Isoxazolo[5,4-*B*]Pyridines; *International Journal of Professional Studies* ; Vol 16, 88-103; DOI: http://doi.org/10.37648/ijps.v16i01.009

great potential in the treatment of cancer [7-8] as it is over expressed in majority of human organs (e.g. lung, breast, colon and ovarian etc.,). In finding new treatments for cancer treatment, EGFR has been chosen as a target.



Anticancer Agent | Iosartan Antihypertensive Agent | Antimicrobial Agent

Inflammation plays a role in healing, but chronic inflammation may increase the risk of various diseases, including some cancers, atherosclerosis, rheumatoid arthritis, periodontitis and hay fever [9]. Chronic inflammation is also referred to as slow, long-term inflammation testing several months to years. Generally the extent and effects of chronic inflammation vary depending on the cause of injury and the ability of body to repair and overcome the damage.

Constant COX-1 activity is required for the physiological role of prostaglandins (PGs) in processes, such as preserving the integrity of the stomsach, mucosa and ensuring proper vascular hemostatis [10]. COX-2, on the other hand, is an inducible enzyme whose expression is triggered by an inflammatory event. Prostaglandin production suppression by cyclooxygenase inhibitory drug is linked to the pharmacological impact of NSAIDs. Due to their effective anti-inflammatory, analgesic, and antipyretic properties, NSAIDs have been extensively utilised in clinical practice.

Exploring techniques for the development and synthesis of various heterocyclic containing isoxazole scaffolds and their applications holds great importance in the realm of medicinal chemistry. Heterocyclic compounds, including isoxazole derivatives, are meticulously examined for their pharmacological action and have emerged as significant pharmacophores [11-12]. These compounds exhibit a wide range of therapeutic uses, including an anticancer properties, anti-inflammatory effects, CNS depressant properties, antimicrobial activity, analgesic effects, antioxidant properties, antitubercular activity, and other biological activities such as GABA agonistic activity, antihypertensive activity, and inhibitory activity [13-16]. The presence of diverse functional groups, such as pyridines, azoles linked to the basic isxoxazole pharmacophoric unit results in different modes of action that may be beneficial for treating various infections [17]. Alternatively, tetrazoles are proved to be potent antimicrobial, antitubercular, hypoglycemic, cycloxygenase inhibitory, anti-inflammatory and anticancer agents [18-22].

Based on these observations, and as a sequel to our work related to isoxazole derivatives synthesis, and biological studies [23-28], we, herein, report the anticancer, and anti-inflammatory activities of a series of novel tetrazole linked isoxazolopyridines.

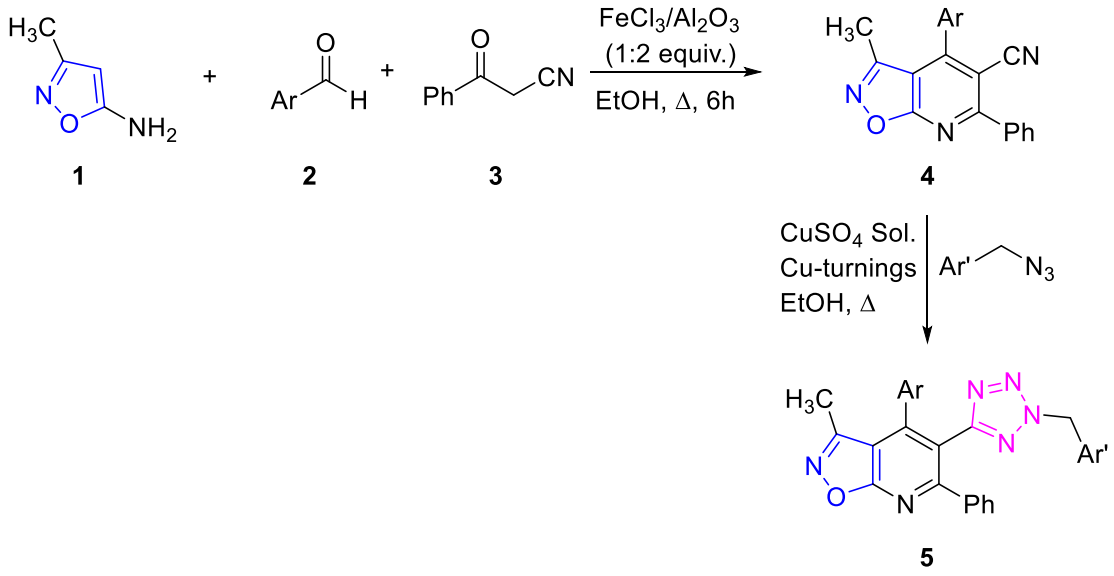
Results and Discussion

Synthesis

The synthesis of title compounds **5a-l** was accomplished by synthetic sequence shown in **Scheme 1**. The three-component reaction of 5-amino-3methylisoxazole **1**, substituted aromatic aldehyde **2**, and benzoyl acetonitrile **3** in refluxing ethanol in presence of $FeCl_3$ on basic alumina (1:2 equiv) as catalyst furnished 3-methyl-4-aryl-6-phenyl isoxazolo[5,4-*b*]pyridine-5-carbonitriles **4** in excellent yields (80-85%). When the same reaction was carried out in refluxing ethanol without catalyst or in the presence of catalysts such as ammonium acetate, acetic acid, *L*-proline or $FeCl_3$ (without alumina) the desired product **4** was obtained in low to moderate yields (15-50%). The rate of reaction was studied with respect to different substituents present on aromatic aldehydes. By TLC monitoring, it was found that different reaction rates (yields) are found with reaction time ranging from 2-10 hours depending on substitution on the benzene ring of aromatic aldehydes **2**. Herein, the electron withdrawing groups including chlorine (**2d** and **2g**)

and bromine (**2h**) led to a faster reaction (2-4 hours). In contrast, substituents such as 2-CH_3 (**2b**), 4-CH_3 (**2c**), 2-OCH_3 (**2e**), 4-OCH_3 (**2f**) served as electron-donating groups, requiring longer duration (6-10 hours). The pure products were obtained in higher yields (60-80%) for **2d**, **2g**, and **2h**, compared to 20-50% yields for **2a**, **2b**, **2c**, **2e**, and **2f (Table 1)**. The effect of different catalysts, and $FeCl_3/Al_2O_3$ ratio was also studied in different solvents. The desired product **4a** was observed in low to moderate yields (30-60%) for the reaction conducted in refluxing ethanol, DMF and CH_3CN with catalysts such as NH4OAc, AcOH, *L*-proline and $FeCl_3$ alone with $Fecl_3$ on basic alumina as catalyst (1:1). When the reaction was carried out with $FeCl_3/Al_2O_3$ (1:2 equiv) in EtOH 85% yield is noticed **(Table 2)**.

Compound **4** on reaction with benzyl azides in presence of saturated copper sulfate solution and cu-turnings in ethanol gave the corresponding 5-(2-benzyl-2*H*-tetrazol-5-yl)-3-methyl-4-aryl-6-phenyl isoxazolo[5,4-*b*]pyridines **5a-l** by [2+3]cycloaddition reaction. The structure of the products **4** and **5** were established on the basis of their micro analytical and spectral (IR, ^{1}H NMR, ^{13}C NMR and MS) data **(Scheme I)**.



Compound	Ar		Compound	Ar	Ar’
4a	C_6H_5		**5a**	C_6H_5	C_6H_5
4b	2-$CH_3C_6H_4$		**5b**	2-$CH_3C_6H_4$	4-$OCH_3C_6H_4$
4c	4-$CH_3C_6H_4$		**5c**	4-$CH_3C_6H_4$	4-$OCH_3C_6H_4$
4d	4-ClC_6H_4		**5d**	4-ClC_6H_4	4-BrC_6H_4
4f	4-$OCH_3C_6H_4$		**5e**	2-$OCH_3C_6H_4$	4-ClC_6H_4
4g	2-ClC_6H_4		**5f**	4-$OCH_3C_6H_4$	4-ClC_6H_4
4h	2-BrC_6H_4		**5g**	2-ClC_6H_4	4-BrC_6H_4
			5h	2-BrC_6H_4	C_6H_5
			5i	C_6H_5	4-$OCH_3C_6H_4$
			5j	C_6H_5	4-ClC_6H_4
			5k	2-ClC_6H_4	C_6H_5
			5l	4-$OCH_3C_6H_4$	C_6H_5

Scheme 1. Synthetic route for targeted compounds 5a-5l

Table 1. Optimization of reaction conditions for the synthesis of compound 4

Entry	Compound	Catalyst	Reaction Time (h)	Yield (%)
1	**4a, C_6H_5**	$FeCl_3/Al_2O_3$	6	40
2	**4b, 2-$CH_3C_6H_4$**	$FeCl_3/Al_2O_3$	8	30
3	**4c, 4-$CH_3C_6H_4$**	$FeCl_3/Al_2O_3$	8	35
4	**4d, 4-ClC_6H_4**	$FeCl_3/Al_2O_3$	2	80
5	**4e, 2-$OCH_3C_6H_4$**	$FeCl_3/Al_2O_3$	6	40
6	**4f, 4-$OCH_3C_6H_4$**	$FeCl_3/Al_2O_3$	8	50
7	**4g, 2-ClC_6H_4**	$FeCl_3/Al_2O_3$	2.5	85
8	**4h, 2-BrC_6H_4**	$FeCl_3/Al_2O_3$	2	85

Table 2. Synthesis of compounds 4a-h in different catalysts and solvents.

Entry	Catalyst	Solvent	Time (h)	Temp (°C)	Yield (%)
1	NH_4OAc (2 equiv)	EtOH	10	80°C	30
2	AcOH (2 equiv)	EtOH	6	80°C	40
3	*L*-proline (2 equiv)	EtOH	6	80°C	45
4	$FeCl_3$ (1 equiv)	EtOH	6	80°C	60
5	$FeCl_3/Al_2O_3$ (1:2 equiv)	EtOH	6	80°C	85
6	$FeCl_3/Al_2O_3$ (1:1 equiv)	EtOH	6	80°C	65
7	$FeCl_3/Al_2O_3$ (1:2 equiv)	DMF	6	100°C	55
8	$FeCl_3/Al_2O_3$ (1:2 equiv)	MeCN	6	80°C	35

IR spectra of **4** exhibited absorption bands at 2210 cm^{-1} due to CN stretching vibration. In ^1H NMR spectra of **4** isoxazole methyl protons appeared as sharp singlet at δ 2.28, whereas aromatic protons shown as complex multiplet between δ6.89-7.80. ^{13}C NMR spectra of **4** is in agreement with the proposed structure. The mass spectrum of **4a** displayed the molecular ion $[M + H]^+$ at *m/z* 312 confirming cyclization. The IR spectra of **5** showed prominent absorption bands at 1530 and 1650 cm^{-1} due to N=N and C=N functional groups stretching vibrations respectively. ^1H NMR spectra of **5** exhibited a singlet at δ 4.59 due to N-CH_2 proton attached to tetrazole moiety confirming cyclization. The ^{13}C NMR spectra of **5** displayed N-CH_2 carbon at δ 59.36. Rest of the signals are in agreement with the proposed structure. In the mass spectrum of **5a** the molecular ion $[M + H]^+$ appeared at *m/z* 445 confirming cyclization. Data from the elemental analyses further confirmed the assigned structures of **4a-h** and **5a-l**.

Biological activity

Anticancer activity

The newly synthesized tetrazole linked isoxazolopyridines **5a-l** were evaluated for *in vitro* anticancer activity against a panel of human cancer cell lines HeLa, EAC and MCF-7 following literature procedures [29-30]. *Cisplatin* (DDP) was used as the reference drug, and the results are summarized in **Table 1**. IC_{50} values were based on dose response curves (IC_{50} value, defined as the concentration corresponding to 50% growth inhibition). Data from **Table 3** indicates that, some of the compounds **5c, 5d, 5f,** and **5l** showed excellent activity against the tumor cells. All the compounds showed a moderate to good anticancer activity against three different cell lines and they are not selective towards any cell lines. The presence of electron donating groups as well as electron withdrawing groups on the benzene rings (**5c, 5d, 5f,** and **5l**) enhanced the anticancer activity and has shown excellent activity, whereas compounds **5b, 5e, 5g, 5h, 5i** and **5k** exhibited good activity may be due to the presence of basic tetrazole linked isoxazolopyridine skeleton. Compound **5a** withour any substituents on benzene ring is least active. Compounds **5c** and **5d** have more cytotoxic activity. The results suggested that, among all the compounds tested **5c,** and **5d** exhibited the most cytotoxic activity in all the three cell lines.

Having obtained the excellent cytotoxic properties of compounds **5c** and **5d** in *in vitro* studies, we evaluated the *in vivo* antitumor properties of these compounds in EAC-bearing mice by using liquid tumor model [31-33]. The effect of the two different doses on compounds **5c** and **5d** (5 mg/kg and 10 mg/kg) on body weight, mean survival

time, % increase life span, tumor volume, packed cell volume, tumor cell count (viable cells) was studied and the results were presented in **Table 4**. The compound **5c** has significant activity ($p < 0.001$) in both doses, decreased the body weight of EAC-bearing mice, significantly increased the mean survival time and deceased the tumor volume, packed cell volume, and viable tumor cell count in both doses. Compound **5d** showed significant activity only in higher dose (10 mg/kg). On the day 14, the biochemical and haematological parameters, as regard to haemoglobin level, erythrocytes and leucocytes counts, were compared with EAC control groups, standard drug *Cisplatin* treated groups and the groups injected with the compounds **5c** and **5d**. The biochemical and haematological parameters in the group treated with the compound **5c** have been nearly recovered completely to the normal values. These results could indicate macrophage activation and inhibition of vascular permeability, with the comparison of tumor control **(Table 5)**. The derivatives **5c** and **5d** increased the haemoglobin and RBC levels and decreased the WBC levels when compared with the tumor control, **5c** decreased these levels more significantly than **5d**. Treatment with the **5c** and **5d** brought back the differential leukocyte count to normal levels. This indicates that the test drugs possess protective action on haemopoeitic system. These results suggest that compound **5c** is more active in *in vivo* cytotoxic studies and by doing simple modification in the structure a potent anticancer drug can be developed.

Table 3. Cytotoxic activities of 5a-j on human cancer cell lines [*in vitro* (IC_{50}, µg/mL[b])].

Compound	Ar	Ar[1]	HeLa	EAC	MCF-7
5a	C_6H_5	C_6H_5	44.32 ± 0.53	40.32 ± 0.28	47.40 ± 0.55
5b	2-$CH_3C_6H_4$	4-$OCH_3C_6H_4$	30.40 ± 0.05	35.12 ± 0.49	40.70 ± 0.39
5c	4-$CH_3C_6H_4$	4-$OCH_3C_6H_4$	10.02 ± 0.30	12.33 ± 0.36	11.55 ± 0.11
5d	4-ClC_6H_4	4-BrC_6H_4	11.40 ± 0.05	10.32 ± 0.43	12.40 ± 0.35
5e	2-$OCH_3C_6H_4$	4-ClC_6H_4	12.35 ± 0.51	11.50 ± 0.60	10.44 ± 0.43
5f	4-$OCH_3C_6H_4$	4-ClC_6H_4	20.25 ± 0.43	22.32 ± 0.35	30.05 ± 0.02
5g	2-ClC_6H_4	4-BrC_6H_4	32.26 ± 0.03	41.05 ± 0.61	33.36 ± 0.49
5h	2-BrC_6H_4	C_6H_5	30.21 ± 0.36	32.61 ± 0.61	35.43 ± 0.43
5i	C_6H_5	4-$OCH_3C_6H_4$	12.25 ± 0.35	18.16 ± 0.65	19.40 ± 0.35
5j	C_6H_5	4-ClC_6H_4	25.00 ± 0.05	20.00 ± 0.23	31.85 ± 0.33
5k	2-ClC_6H_4	C_6H_5	30.38 ± 0.08	48.05 ± 0.32	42.40 ± 0.54
5l	4-$OCH_3C_6H_4$	C_6H_5	10.20 ± 0.04	15.25 ± 0.33	10.66 ± 0.35
Cisplatin (DDP)	-	-	3.84	4.81	4.19

[a]Cytotoxicity as IC_{50} for each cell line, is the concentration of compound which reduced by 50% the optical density of treated cells with respect to untreated cells using the MTT assay.

[b]Data represent the mean values of three independent determinations.

Table 4. Anticancer activity of 5c & 5d on EAC-bearing mice

Parameter	EAC control (5 x 106 cells)	*Cisplatin* (5 mg/kg)	5c (5 mg/kg)	5c (10 mg/kg)	5d (5 mg/kg)	5d (10 mg/kg)
Body weight (g)	11.20 ± 0.55	2.98 ± 0.33***	9.41 ± 0.30*	6.70 ± 0.48 ***	9.00 ± 0.25[ns]	7.89 ± 0.33***
Mean survival time (days)	12.70 ± 0.02	19.30 ± 0.36***	17.40 ± 0.67**	20.10 ± 0.03***	12.80 ± 0.32*	16.00 ± 0.53***
% increase in life span (% ILS)	-	13.86***	17.70*	41.31***	8.02*	21.38**

Tumor Volume (mL)	9.10 ± 0.89	2.01 ± 0.35***	5.61 ± 0.61***	6.50 ± 0.39***	8.69 ± 0.54^{ns}	6.29 ± 0.22**
Packed cell volume (mm)	1.75 ± 0.49	0.25 ± 0.05***	1.25 ± 0.23*	0.94 ± 0.11***	2.13 ± 0.33^{ns}	1.69 ± 0.22^{ns}
Viable tumor cell count (x10^7 cells/mL)	5.39 ± 0.51	0.16 ± 0.04***	3.05 ± 0.35***	2.27 ± 0.43***	6.21 ± 0.46^{ns}	4.07 ± 0.44***

n = 10 and all values were expressed as mean ± SEM, ***P < 0.001, **P < 0.005, *P < 0.05, ^{ns} non-significant, compared to tumor control.

Table 5. Effect of 5c & 5d on biochemical and haematological parameters in EAC-bearing mice.

Treatment	**Parameters**					
	Haemoglobin (mg %)	RBC (million/mm^3)	WBC (10^3 cells/mm^3)	Lymphocytes (%)	Neutrophils (%)	Monocytes (%)
Normal	12.10 ± 0.662	3.512 ± 0.084	6.160 ± 0.120	58 ± 1.31	19 ± 1.46	1 ± 0.29
Tumor Control	4.750 ± 0.455	2.740 ± 0.081	10.60 ± 0.545	20 ± 0.567	63 ± 1.25	2.2 ± 0.12
Cisplatin (5mg/kg)	1.50 ± 1.025***	3.190 ± 0.087***	8.160 ± 0.231***	53 ± 0.782***	15 ± 1.17***	0.9 ± 0.16***
5c (5 mg/kg)	6.080 ± 0.86*	2.020 ± 0.066**	11.48 ± 0.355^{ns}	21 ± 0.936**	30 ± 0.85*	1 ± 0.36*
5c (10 mg/kg)	8.30 ± 1.103**	2.880 ± 0.086**	16.92 ± 0.476***	39 ± 0.759**	35 ± 0.97***	1.2 ± 0.18**
5d (5mg/kg)	3.960 ± 0.636^{ns}	1.720 ± 0.086^{ns}	10.90 ± 0.343^{ns}	25 ± 0.583*	30 ± 0.48*	1.8 ± 0.46^{ns}
5d (10 mg/kg)	6.020 ± 0.797*	2.300 ± 0.158*	19.80 ± 0.293**	30 ± 0.857**	41 ± 0.84**	2.2 ± 0.53*

All values were expressed as mean ± SEM (*n* = 10), *P < 0.05, **P < 0.01, ***P < 0.001, ^{ns} non-significant, compared to tumor control.

Anti-inflammatory activity

The anti-inflammatory activity of the title compounds, **5a-l** were evaluated by carrageenan-induced paw edema method in rats [34] at a dose of 100 mg/kg body weight using *Ibuprofen* as a reference drug. Results were expressed as a mean ± S.E. The anti-inflammatory properties were recorded at successive intervals of 0, 1, 2, 4 and 6 h and compared with that of standard drug *Ibuprofen*. The anti-inflammatory activity data **(Table 6)** indicated that all the compounds **5a-l** exhibited significant activity by decreasing the paw volume that was produced by carrageenan. Among all the compounds **5a-l** tested, it is interesting to note that the compounds **5c, 5d, 5f** and **5l** showed better anti-inflammatory activity. This may be due to the presence of electron donating and electron withdrawing substitutions on the benzene ring, besides the presence of tetrazole linked isoxazolo[5,4-*b*]pyridine ring frame work. Compounds **5b, 5e, 5g, 5h, 5i** and **5k** did not influence the activity much. Among the all the compounds **5a** is least active. The preliminary *in vitro* cyclo oxygenase inhibition studies [35] of tetrazole linked isoxazolopyridines **5a-l** have evidenced that **5c, 5d, 5f** and **5l** are more selective and active than compounds **5b, 5e, 5g, 5h, 5i** and **5k**. The compounds **5c** and **5d** having methyl and methoxy, chloro and bromo groups on benzene ring showed better activity and selective towards cyclooxygenase-2 enzyme. Compounds **5b, 5e, 5g, 5h, 5i** and **5k** showed moderate anti-inflammatory activity but they are less selective COX-2 inhibitors, when compared with standard drug *Ibuprofen*. The possible improvement of selective COX-2 inhibitory activity of this basic isoxazolo[5,4-*d*] pyridine linked with tetrazole ring, may be possible through modulation of ring substituents and/or additional functionalization which requires further investigation **(Table 6).**

Table 6. Anti-inflammatory activity of 5a-l.

Paw volume (mL of Hg)[b]					
Compound[a]	**0 h**	**1 h**	**2 h**	**4 h**	**6 h**
5a	0.34 ± 0.02	0.75 ± 0.04	0.76 ± 0.06^{ns}	0.72 ± 0.05**	0.59 ± 0.04**
5b	0.35 ± 0.02	0.74 ± 0.04	0.84 ± 0.04^{ns}	0.78 ± 0.03**	0.64 ± 0.02**
5c	0.21 ± 0.05	0.40 ± 0.02	0.58 ± 0.03	0.55 ± 0.04*	0.41 ± 0.02**
5d	0.20 ± 0.06	0.31 ± 0.05	0.48 ± 0.02	0.51 ± 0.03	0.60 ± 0.02**
5e	0.34 ± 0.03	0.82 ± 0.01	0.93 ± 0.04^{ns}	0.80 ± 0.02**	0.61 ± 0.03**
5f	0.22 ± 0.04	0.34 ± 0.02	0.48 ± 0.02	0.62 ± 0.04**	0.65 ± 0.04**

5g	0.38 ± 0.04	0.76 ± 0.06	0.81 ± 0.02[ns]	0.62 ± 0.04*	0.58 ± 0.04**
5h	0.31 ± 0.04	0.71 ± 0.02	0.62 ± 0.04	0.78 ± 0.03*	0.42 ± 0.03**
5i	0.21 ± 0.01	0.33 ± 0.04	0.45 ± 0.02[ns]	0.57 ± 0.03**	0.31 ± 0.03***
5j	0.28 ± 0.03	0.62 ± 0.04	0.52 ± 0.03	0.81 ± 0.03**	0.43 ± 0.04***
5k	0.36 ± 0.04	0.52 ± 0.03	0.54 ± 0.03[ns]	0.49 ± 0.04*	0.54 ± 0.03***
5l	0.23 ± 0.04	0.32 ± 0.01	0.59 ± 0.05	0.34 ± 0.03**	0.33 ± 0.01***
Control	0.36 ± 0.3	0.90 ± 0.05	1.07 ± 0.08	1.2 ± 0.05	0.96 ± 0.03
Ibuprofen	0.30 ± 0.5	0.66 ± 0.03*	0.70 ± 0.05***	0.60 ± 0.05***	0.40 ± 0.05***

Statistically significant compound to respective control value *P < 0.05, **P < 0.01, ***P < 0.001.
[a]Dose levels: test compound (100mg/kg b.wt) ibuprofen (100 mg/kg b.wt.)
[b]Values are expressed as mean ± SE.
[ns]Nonsignificant, campared to control.
[#]n = 6, number of animals used in each group.

Table 7. *In vitro* cyclooxygenase inhibition studies of 5a-l.

Compound[c]	**%inhibition[a,b]**	
	COX-1 (10 µM)	**COX-2 (10 µM)**
5a	30.4	32.4
5b	28.2	29.3
5c	18.3	80.3
5d	17.8	86.1
5e	38.7	41.1
5f	22.2	48.6
5g	38.4	36.4
5h	34.6	32.1
5i	19.3	72.7
5j	42.2	84.2
5k	26.5	69.4
5l	16.4	79.3
Celecoxib	2.8	100

[a]Values are acquired using *in vitro* ovine COX-1/COX-2 assay kit (Catalog No. 760112, Cayman Chemicals Inc., Ann Arbor, MI).
[b]Experiments were carried out in duplicate and have less than 10% error.
[c]Test compounds and Celecoxib were administered orally at the dose of 10 mg/kg.

Molecular Docking Studies

The potential macromolecular targets for the synthesized compounds were identified utilizing the Binding DB, and SWISS Target Prediction tool. A range of proteins implicated in cancer pathophysiology, such as Epidermal Growth Factor Receptor (5CNN), in addition to other recognized target proteins, were examined. Predictions indicate that the synthesized analogs may effectively inhibit 5CNN specifically.

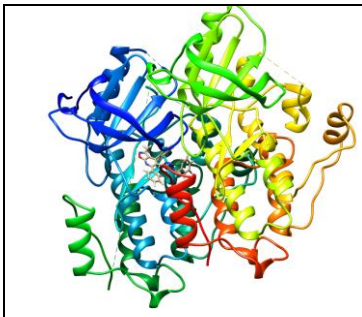
(IJPS) 2023, Vol. No. 16, Issue 1, Jul-Dec **e-ISSN: 2455-6270 p-ISSN: 2455-7455**

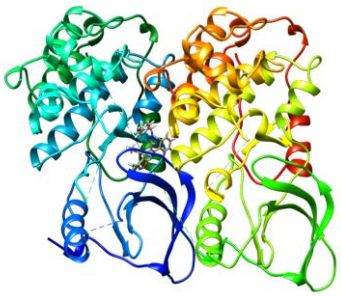
The compounds **5a–l** analyzed demonstrated an Autodock with binding score maximum of -9.5371 K.cal/mol for compound **5c**, -9.3969 for compound **5d**, -9.3097 for compound **5f**, and -9.2564 for compound **5l** against EGFR (5CNN). The potential affinity of these compounds was validated by examining the binding cavities of the target proteins. To validate the docking methodology, a docking simulation was conducted comparing the drawn structure of the *Gefitinib* within the protein. It was observed that certain synthesized derivatives (**5c, 5d, 5f** and **5l**) exhibited binding scores exceeding that of the *Gefitinib*, which were recorded at -9.5371 (**5c**), -9.3969 (**5d**), -9.3097 (**5f**) and -9.2564 (**5l**) K.cal/mole for the same enzyme (-9.1638).

Molecular modelling experiments have revealed the importance of the interactions of the amino acid residues with the docking ligands, the synthesized compounds **5a–l** showed docking scores in the range of -8.0139 to -9.5371. Overall, four compounds showed docking scores higher than that of the standard. The best score of -9.5371 throughout the series of compounds was displayed by **5c**, which has a tolyl and methoxy substitution at 4th position of aromatic rings. The next was **5d** which has docking score of -9.3969, with the presence of chloro and bromo groups at 4th position of aromatic rings. The docking results are in sync with the observed anticancer and anti-inflammatory activities.

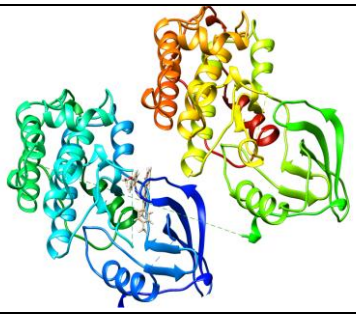
Table 8. Docking interactions of ligands for EGFR (5CNN).

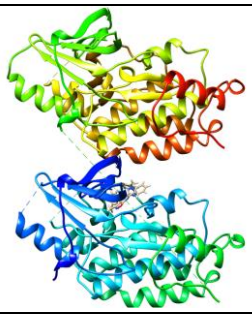
Compound	Key Residue	No. of Hydrogen Bonds	Docking Score(Kcal/Mol)
5a	Ser; Val; Cy	3	-8.8014
5b	Asp; Asp; Arg	3	-9.0995
5c	Arg; Ser; Ala	6	-9.5371
5d	Met; Thr; Pro	3	-9.3969
5e	Phe; Arg; Lys	3	-9.0139
5f	Gly; Thr; Asp	6	-9.3097
5g	Met; Thr; Pro	3	-9.1501
5h	Arg; Arg; Cys	3	-8.8664
5i	Leu; Arg	2	-9.0817
5j	Arg; Leu; Val	3	-9.0528
5k	Met; Arg; Leu	3	-9.1587
5l	Leu; Cys; Asp	3	-9.2564
Gefitinib	Gly, Thr, Asp, Cys, Met; Lys	6	-9.1638

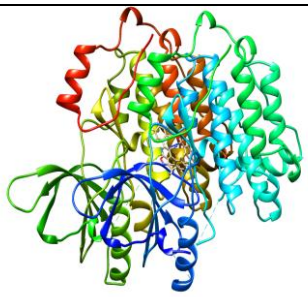
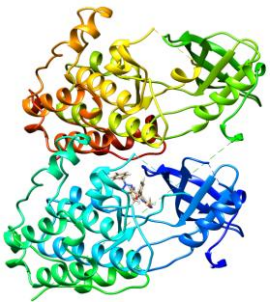




5a **5b**





5c	5d
	
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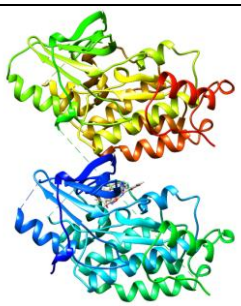
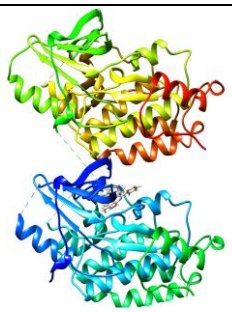
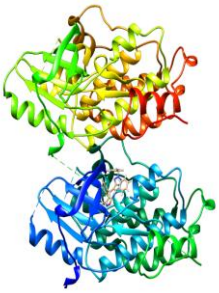
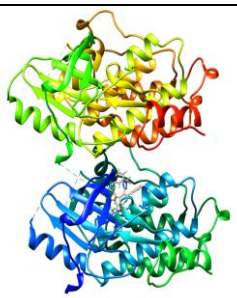
	
5g	**5h**
	
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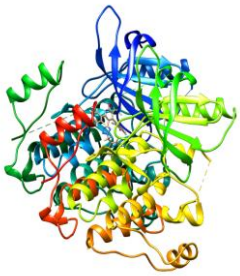
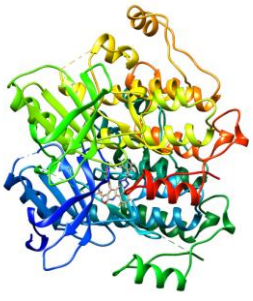
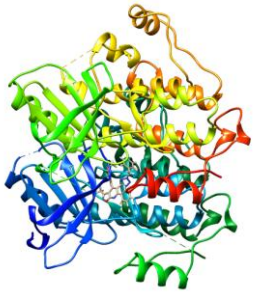
	
5k	**5l**
	
Gefitinib	

EXPERIMENTAL

Chemistry

All the melting points were determined on a Fisher-Johns melting point apparatus, and are uncorrected. Analytical TLC was performed on Merck precoated 60 F254 silica gel plates. Visualization was carried out by exposure to iodine vapour. IR spectra (KBr pellet) were recorded on a Perkin-Elmer BX series FT-IR spectrometer. ^{1}H NMR spectra were recorded on a Varian Gemini 300 MHz spectrometer. ^{13}C NMR spectra were recorded on a Bruker 75 MHz spectrometer. Chemical shift values are given in δ(ppm) with tetramethyl silane as an internal standard. ESI mass spectra were recorded on a Agilent LC-MSD mass spectrometer. Elemental analyses were performed on a Carlo Erba 106 and Perkin-Elmer model 240 analyzers.

Synthesis

Preparation of the catalyst ($FeCl_3$/basic Al_2O_3). A mixture of hydrated Ferric chloride ($FeCl_3.6H_2O$, 5 g) and basic Al_2O_3 (10 g) in acetone (35 mL) was stirred at room temperature for 2 hours, and then concentrated under reduced pressure. The resulting yellow powder was dried at 100^{o}C under reduced pressure for 2 h [43].

General procedure for the synthesis of 3-methyl-4-aryl-6-phenylisoxazolo[5,4-*b*]pyridine-5-carbonitriles (4a-h). To a mixture of 5-amino-3-methylisoxazole **1** (1 mmol), arylaldehyde **2** (1 mmol), benzoyl acetonitrile **3** (1 mmol) was added $FeCl_3$/basic Al_2O_3 (1 mmol; 1:2 ratio) in anhydrous ethanol (20 mL), and the contents are heated under reflux for 6h. After the completion of the reaction, as indicated by TLC, the reaction mixture was diluted with ethyl acetate, and the insoluble solid was filtered. The solvent was evaporated under reduced pressure and the product was recrystallized from pet. ether and benzene.

3-Methyl-4,6-diphenylisoxazolo[5,4-*b*]pyridine-5-carbonitrile (4a). Pale Yellow solid, mp: 140-142^{o}C, IR spectrum, *v,* cm^{-1}: 2210 (CN); ^{1}H NMR spectrum (300 MHz, $CDCl_3$), δ, (ppm): 2.28 (s, 3H, isoxazole CH_3), 6.89-7.80 (m, 10H, Ar-H); ^{13}C NMR (75MHz, $CDCl_3$), δ, (ppm): 12.84, 100.52, 107.32, 117.25, 126.32, 127.39, 128.21, 129.32, 130.21, 131.52, 132.01, 133.25, 134.52, 136.21, 140.10, 142.35, 152.36, 156.31, 167.21, 169.45; Mass spectrum: *m/z* 312 $[M+H]^+$. Found, %: C, 77.14; H, 4.14; N, 13.45; $C_{20}H_{13}N_3O$, Calculated, %: C, 77.17; H, 4.16; N, 13.46.

3-Methyl-6-phenyl-4-(2-methylphenyl)isoxazolo[5,4-*b*]pyridine-5-carbonitrile (4b). Pale Yellow solid, mp: 149-151^{o}C; IR spectrum, *v,* cm^{-1}: 2215 (CN); ^{1}H NMR spectrum (300 MHz, $CDCl_3$), δ, (ppm): 2.30(s, 3H, isoxazole CH_3), 2.50 (s,3H, Ar-CH_3), 6.91-7.83 (m, 9H, Ar-H); ^{13}C NMR (75MHz, $CDCl_3$), δ, (ppm): 12.80, 21.56, 101.12, 108.30, 116.05, 125.92, 126.29, 127.11, 128.22, 129.81, 132.22, 133.21, 134.65, 135.50, 136.31, 139.36, 141.25, 151.26, 155.41, 168.11, 170.05; Mass spectrum: *m/z* 326 $[M+H]^+$. Found, %: C, 77.52; H, 4.60; N, 12.93. $C_{21}H_{15}N_3O$. Calculated, %: C, 77.53; H, 4.61; N, 12.92.

3-Methyl-6-phenyl-4-(4-methylphenyl)isoxazolo[5,4-*b*]pyridine-5-carbonitrile (4c). Pale Yellow solid, mp: 158-160^{o}C; IR spectrum, *v,* cm^{-1}: 2213 (CN); ^{1}H NMR spectrum (300 MHz, $CDCl_3$), δ, (ppm): 2.32 (s, 3H, isoxazole CH_3), 2.52 (s,3H, Ar-CH_3), 6.90-7.91 (m, 9H, Ar-H); ^{13}C NMR (75MHz, $CDCl_3$), δ, (ppm): 12.78, 22.06, 102.02, 107.60, 117.25, 124.52, 125.89, 126.31, 127.82, 128.61, 131.20, 132.81, 135.35, 136.52, 137.41, 138.96, 142.15, 150.86, 154.31, 167.17, 169.25; Mass spectrum: *m/z* 326 $[M+H]^+$. Found, %: C, 77.51; H, 4.59; N, 12.90. $C_{21}H_{15}N_3O$. Calculated, %: C, 77.53; H, 4.61; N, 12.92.

4-(4-Chlorophenyl)-3-methyl-6-phenylisoxazolo[5,4-*b*]pyridine-5-carbonitrile (4d). Pale Yellow solid, mp: 185-187^{o}C. IR spectrum, *v,* cm^{-1}: 2212 (CN); ^{1}H NMR spectrum (300 MHz, $CDCl_3$), δ, (ppm): 2.30 (s, 3H, isoxazole CH_3), 6.87-7.92 (m, 9H, Ar-H); ^{13}C NMR (75MHz, $CDCl_3$), δ, (ppm): 12.79, 101.92, 108.62, 118.65, 127.22, 128.30, 129.31, 130.12, 131.23, 132.52, 133.21, 134.35, 135.50, 137.01, 139.12, 143.31, 151.46, 157.31, 168.61, 170.15; Mass spectrum: *m/z* 346 $[M+H]^+$. Found, %: C, 69.53; H, 3.46; N, 12.16. $C_{20}H_{12}ClN_3O$. Calculated, %: C, 69.56; H, 3.47; N, 12.17.

4-(2-Methoxyphenyl)-3-methyl-6-phenylisoxazolo[5,4-*b*]pyridine-5-carbonitrile (4e). Pale Yellow solid, mp: 166-168°C; IR spectrum, *v,* cm^{-1}: 2213 (CN); ^1H NMR spectrum (300 MHz, $CDCl_3$), δ, (ppm): 2.30 (s, 3H, isoxazole CH_3), 3.68 (s., 3H, Ar-OCH_3), 6.93-7.95 (m, 9H, Ar-H); ^{13}C NMR (75MHz, $CDCl_3$), δ, (ppm): 12.80, 56.98, 103.12, 106.80, 118.66, 123.92, 124.92, 127.11, 128.45, 129.31, 130.80, 133.21, 136.51, 137.34, 138.22, 139.16, 143.05, 151.86, 154.31, 167.65,169.25; Mass spectrum: *m/z* 342 $[M+H]^+$. Found, %: C, 73.92; H, 4.38; N, 12.33. $C_{21}H_{15}N_3O_2$. Calculated, %: C, 73.90; H, 4.39; N, 12.31.

4-(4-Methoxyphenyl)-3-methyl-6-phenylisoxazolo[5,4-*b*]pyridine-5-carbonitrile (4f). Pale Yellow solid; mp 173-175°C. IR spectrum, *v,* cm^{-1}: 2216 (CN); ^1H NMR spectrum (300 MHz, $CDCl_3$), δ, (ppm): 2.32 (s, 3H, isoxazole CH_3), 3.66 (s, 3H, Ar-OCH_3), 6.96-7.97 (m, 9H, Ar-H); ^{13}C NMR (75MHz, $CDCl_3$), δ, (ppm): 12.82, 56.23, 104.22, 107.62, 119.62, 124.32, 125.66, 128.31, 129.43, 130.21, 131.20, 134.22, 136.56, 138.32, 139.23, 140.06, 142.09, 150.36, 155.21, 168.25, 170.35; Mass spectrum: *m/z* 342 $[M+H]^+$. Found, %: C, 73.92; H, 4.38; N, 12.30. $C_{21}H_{15}N_3O_2$. Calculated, %: C, 73.90; H, 4.39; N, 12.31.

4-(2-Chlorophenyl)-3-methyl-6-phenylisoxazolo[5,4-*b*]pyridine-5-carbonitrile (4g). Pale Yellow solid, mp: 189-191°C. IR spectrum, *v,* cm^{-1}: 2212 (CN); ^1H NMR spectrum (300 MHz, $CDCl_3$), δ, (ppm): 2.30 (s, 3H, isoxazole CH_3), 6.90-7.95 (m, 9H, Ar-H); ^{13}C NMR (75MHz, $CDCl_3$), δ, (ppm): 12.81, 102.22, 109.32, 119.25, 128.23, 129.50, 130.36, 131.15, 132.33, 133.22, 134.20, 135.35, 136.20, 138.21, 140.02, 142.91, 150.43, 158.21, 169.11, 170.36; Mass spectrum: *m/z* 346 $[M+H]^+$. Found, %: C, 69.58; H, 3.45; N, 12.15. $C_{20}H_{12}ClN_3O$. Calculated, %: C, 69.56; H, 3.47; N, 12.17.

4-(2-Bromophenyl)-3-methyl-6-phenylisoxazolo[5,4-*b*]pyridine-5-carbonitrile (4h) Brown solid, mp: 198-200°C; IR spectrum, *v,* cm^{-1}: 2215 (CN); ^1H NMR spectrum (300 MHz, $CDCl_3$), δ, (ppm): 2.28 (s, 3H, isoxazole CH_3), 6.94-7.91 (m, 9H, Ar-H); ^{13}C NMR (75MHz, $CDCl_3$), δ, (ppm): 12.78, 101.42, 108.62, 118.65, 127.63, 128.60, 129.86, 131.15, 133.63, 134.12, 134.90, 135.55, 137.50, 138.51, 141.32, 143.41, 151.23, 159.26, 168.91, 169.66; Mass spectrum: *m/z* 390 $[M+H]^+$. Found, %: C, 61.66; H, 3.07; N, 10.77. $C_{20}H_{12}BrN_3O$. Calculated, %: C, 61.69; H, 3.08; N, 10.79.

General procedure for the synthesis of 5-(2-benzyl-2H-tetrazol-5-yl)-3-methyl-4,6-diphenylisoxazolo[5,4-b]pyridine (5a-j). To a solution of compound **5** (1 mmol) in ethanol, benzyl azides (1 mmol), saturated copper sulphate solution (10 mL) and Cu turnings (10 mg) was added. The resultant mixture was refluxed for 6h. the reaction was monitored with TLC. After completion of the reaction as indicated by TLC, the reaction mixture was filtered through celite. The celite pad was worked with ethyl acetate, and the combined organic volatiles was removed under reduced pressure. The residue was purified by column chromatography. The product was eluted with hexane-ethyl acetate (7:3).

5-(2-Benzyl-2*H*-tetrazol-5-yl)-3-methyl-4,6-diphenylisoxazolo[5,4-*b*]pyridine (5a). Pale Yellow solid, mp 190-192°C; IR spectrum, *v,* cm^{-1}: 1530 (N=N), 1650 (C=N); ^1H NMR spectrum (300 MHz, $CDCl_3$), δ, (ppm): 2.28 (s, 3H, isoxazole CH_3), 4.59 (s, 2H, N-CH_2),6.98-7.80 (m, 15H, Ar-H); ^{13}C NMR (75MHz, $CDCl_3$), δ, (ppm): 12.85, 59.36, 100.58, 123.68, 124.21, 125.62, 126.11, 128.35, 129.21, 130.21, 131.24, 133.25, 134.69, 136.25, 138.25, 140.21, 142.35, 145.36, 146.01, 147.25, 149.36, 152.32, 154.11, 155.65, 157.32, 165.23, 169.27; Mass spectrum: *m/z* 445$[M+H]^+$. Found, %: C, 72.95, H, 4.51; N, 18.93. $C_{27}H_{20}N_6O$ Calculated, %: C, 72.97, H, 4.50; N, 18.91.

5-(2-(4-Methoxybenzyl)-2*H*-tetrazol-5-yl)-3-methyl-6-phenyl-4-(2-methylphenyl)isoxazolo[5,4-*b*]pyridine (5b). Pale Yellow solid, mp 232-234°C; IR spectrum, *v,* cm^{-1}: 1535 (N=N), 1653 (C=N); ^1H NMR spectrum (300 MHz, $CDCl_3$), δ, (ppm): 2.30 (s, 3H, isoxazole CH_3), 2.50 (s, 3H, Ar- CH_3), 3.68 (s, 3H, Ar-OCH_3), 4.65 (s, 2H, N-CH_2),6.92-7.89 (m, 13H, Ar-H); ^{13}C NMR (75MHz, $CDCl_3$), δ, (ppm): 12.80, 21.92, 56.85, 60.21, 101.18, 122.75, 123.71, 127.22, 128.01, 129.75, 130.20, 131.11, 132.54, 134.15, 135.62, 136.44, 139.65, 141.41, 143.32, 146.76, 147.21, 148.55, 150.16, 153.22, 155.31, 156.35, 158.62, 166.43, 170.07; Mass spectrum: *m/z* 489$[M+H]^+$. Found, %: C, 71.33, H, 4.92; N, 17.23. $C_{29}H_{24}N_6O_2$ Calculated, %: C, 71.31, H, 4.91; N, 17.21.

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5-(2-(4-Methoxybenzyl)-2*H*-tetrazol-5-yl)-3-methyl-6-phenyl-4-(4methylphenyl)isoxazolo[5,4-*b*]pyridine (5c). Pale Yellow solid, mp 226-228ºC; IR spectrum, *v,* cm^{-1}: 1530 (N=N), 1655 (C=N); ^{1}H NMR spectrum (300 MHz, $CDCl_3$), δ, (ppm): 2.28 (s, 3H, isoxazole CH_3), 2.52 (s, 3H, Ar- CH_3), 3.66 (s, 3H, Ar-OCH_3), 4.60 (s, 2H, N-CH_2), 6.97-7.86 (m, 13H, Ar-H); ^{13}C NMR (75MHz, $CDCl_3$), δ, (ppm): 12.75, 22.22, 55.95, 61.11, 102.08, 123.72, 124.70, 127.62, 129.11, 130.15, 131.22, 132.41, 133.24, 135.35, 136.40, 137.65, 140.15, 142.11, 144.22, 146.72, 148.31, 149.65, 151.36, 152.62, 157.11, 158.65, 159.32, 166.22, 169.02; Mass spectrum: *m/z* 489 $[M+H]^+$. Found, %: C, 71.30, H, 4.92; N, 17.20. $C_{29}H_{24}N_6O_2$ Calculated, %: C, 71.31, H, 4.91; N, 17.21.

5-(2-(4-Bromobenzyl)-2*H*-tetrazol-5-yl)-4-(4-chlorophenyl)-3-methyl-6-phenylisoxazolo[5,4-*b*]pyridine (5d). Brown solid, mp 272-274ºC. IR spectrum, *v,* cm^{-1}: 1535 (N=N), 1658 (C=N), ^{1}H NMR spectrum (300 MHz, $CDCl_3$), δ, (ppm): 2.30 (s, 3H, isoxazole CH_3), 4.63 (s, 2H, N-CH_2), 6.99-7.88 (m, 13H, Ar-H); ^{13}C NMR (75MHz, $CDCl_3$), δ, (ppm): 12.76, 56.25, 101.38, 122.88, 125.55, 126.69, 127.32, 129.45, 130.71, 131.61, 132.44, 134.65, 135.60, 137.15, 139.20, 141.01, 143.31, 146.40, 146.01, 148.23, 150.06, 153.30, 155.21, 156.75, 158.11, 166.20, 170.11; Mass spectrum: *m/z* 557 $[M+H]^+$. Found, %: C, 58.25, H, 3.22; N, 15.12. $C_{27}H_{18}BrClN_6O$ Calculated, %: C, 58.27, H, 3.23; N, 15.10.

5-(2-(4-Chlorobenzyl)-2*H*-tetrazol-5-yl)-4-(2-methoxyphenyl)-3-methyl-6-phenylisoxazolo[5,4-*b*]pyridine (5e). Pale Yellow solid; mp 249-251ºC; IR spectrum, *v,* cm^{-1}: 1530 (N=N), 1650 (C=N), ^{1}H NMR spectrum (300 MHz, $CDCl_3$), δ, (ppm): 2.30 (s, 3H, isoxazole CH_3), 3.68 (s, 3H, OCH_3), 4.58 (s, 2H, N-CH_2), 6.91-7.95 (m, 13H, Ar-H); ^{13}C NMR (75MHz, $CDCl_3$), δ, (ppm): 12.78, 56.25, 60.23, 101.65, 120.98, 125.98, 126.81, 129.31, 131.43, 132.69, 133.65, 134.62, 136.65, 137.60, 139.06, 140.04, 140.36, 145.31, 146.70, 148.31, 150.02, 152.12, 155.69, 157.03, 158.32, 160.55, 168.32, 170.19; Mass spectrum: *m/z* 509 $[M + H]^+$. Found, %: C, 66.11, H, 4.16; N, 16.56. $C_{28}H_{21}ClN_6O_2$ Calculated, %: C, 66.14, H, 4.13; N, 16.53.

5-(2-(4-Chlorobenzyl)-2*H*-tetrazol-5-yl)-4-(4-methoxyphenyl)-3-methyl-6-phenylisoxazolo[5,4-*b*]pyridine (5f). Pale Yellow solid, mp 256-258ºC; IR spectrum, *v,* cm^{-1}: 1534 (N=N), 1653 (C=N); ^{1}H NMR spectrum (300 MHz, $CDCl_3$), δ, (ppm): 2.29 (s, 3H, isoxazole CH_3), 3.67 (s, 3H, OCH_3), 4.61 (s, 2H, N-CH_2), 6.88-7.92 (m, 13H, Ar-H); ^{13}C NMR (75MHz, $CDCl_3$), δ, (ppm): 12.93, 57.05, 61.13, 102.15, 119.93, 126.28, 127.31, 130.36, 132.73, 133.68, 134.25, 135.22, 137.61, 138.66, 140.06, 141.14, 143.36, 146.21, 148.36, 149.36, 151.36, 153.36, 156.79, 158.03, 159.39, 161.15, 169.30, 170.55; Mass spectrum: *m/z* 509 $[M + H]^+$. Found, %: C, 66.17, H, 4.11; N, 16.52. $C_{28}H_{21}ClN_6O_2$ Calculated, %: C, 66.14, H, 4.13; N, 16.53.

5-(2-(4-Bromobenzyl)-2*H*-tetrazol-5-yl)-4-(2-chlorophenyl)-3-methyl-6-phenylisoxazolo[5,4-*b*]pyridine (5g). Brown solid, mp 282-284ºC; IR spectrum, *v,* cm^{-1}: 1531 (N=N), 1652 (C=N); ^{1}H NMR spectrum (300 MHz, $CDCl_3$), δ, (ppm): 2.28 (s, 3H, isoxazole CH_3), 4.60 (s, 2H, N-CH_2),6.85-7.91 (m, 13H, Ar-H); ^{13}C NMR (75MHz, $CDCl_3$), δ, (ppm): 12.91, 57.05, 100.21, 121.98, 126.65, 127.21, 128.35, 130.42, 131.76, 132.38, 133.68, 135.38, 136.30, 138.11, 139.24, 141.11, 144.21, 145.69, 147.21, 149.22, 151.16, 154.25, 156.23, 157.72, 159.32, 167.22, 169.69; Mass spectrum: *m/z* 557 $[M+H]^+$. Found, %: C, 58.24, H, 3.21; N, 15.11. $C_{27}H_{18}BrClN_6O$ Calculated, %: C, 58.27, H, 3.23; N, 15.10.

5-(2-benzyl-2*H*-tetrazol-5-yl)-4-(2-bromophenyl)-3-methyl-6-phenylisoxazolo[5,4-*b*]pyridine (5h). Brown solid, mp: 265-267ºC; IR spectrum, *v,* cm^{-1}: 1536 (N=N), 1650 (C=N), ^{1}H NMR spectrum (300 MHz, $CDCl_3$), δ, (ppm): 2.26 (s, 3H, isoxazole CH_3), 4.58 (s, 2H, N-CH_2), 6.80-7.88 (m, 14H, Ar-H); ^{13}C NMR (75MHz, $CDCl_3$), δ, (ppm): 12.88, 62.02, 99.36, 122.03, 125.61, 126.55, 129.25, 131.43, 132.76, 133.55, 134.63, 136.87, 137.10, 139.32, 140.04, 142.10, 143.22, 144.79, 146.32, 150.23, 152.18, 155.25, 156.83, 157.72, 158.30, 168.11, 169.36; Mass spectrum: *m/z* 523 $[M + H]^+$. Found, %: C, 62.04, H, 3.61; N, 16.07. $C_{27}H_{19}BrN_6O$ Calculated, %: C, 62.06, H, 3.63; N, 16.09.

5-(2-(4-Methoxybenzyl)-2*H*-tetrazol-5-yl)-3-methyl-4,6-*di*phenylisoxazolo[5,4-*b*]pyridine (5i). Pale Yellow solid, mp 241-243ºC; IR spectrum, *v,* cm^{-1}: 1532 (N=N), 1650 (C=N); ^{1}H NMR spectrum (300 MHz, $CDCl_3$), δ, (ppm): 2.30 (s, 3H, isoxazole CH_3), 3.68 (s, 3H, OCH_3), 4.63 (s, 2H, N-CH_2),6.90-7.95 (m, 14H, Ar-H); ^{13}C NMR

(75MHz, $CDCl_3$), δ, (ppm): 12.89, 56.25, 60.33, 103.25, 120.92, 125.68, 127.39, 131.30, 132.91, 134.62, 135.35, 136.36, 138.65, 139.20, 141.71, 142.34, 144.32, 147.26, 148.55, 150.06, 151.65, 153.69, 157.71, 158.25, 159.69, 162.05, 169.66, 171.21; Mass spectrum: *m/z* 475 $[M + H]^+$, Found, %: C, 70.86, H, 4.63; N, 17.70. $C_{28}H_{22}N_6O_2$ Calculated, %: C, 70.88, H, 4.64; N, 17.72.

5-(2-(4-Chlorobenzyl)-2*H*-tetrazol-5-yl)-3-methyl-4,6-diphenylisoxazolo[5,4-*b*]pyridine (5j). Pale Yellow solid, mp 255-257°C: IR spectrum, *v,* cm^{-1}: 1536 (N=N), 1658 (C=N); ^1H NMR spectrum (300 MHz, $CDCl_3$), δ, (ppm): 2.28 (s, 3H, isoxazole CH_3), 4.61 (s, 2H, N-CH_2), 6.95-7.98 (m, 14H, Ar-H); ^{13}C NMR (75MHz, $CDCl_3$), δ, (ppm): 12.91, 61.20, 100.24, 119.88, 126.69, 127.21, 128.65, 130.45, 133.67, 134.60, 135.66, 136.65, 138.25, 139.67, 140.14, 141.31, 144.52, 146.91, 149.21, 151.22, 153.11, 156.58, 158.11, 159.33, 161.15, 169.21, 170.55. Mass spectrum: *m/z* 479 $[M + H]^+$. Found, %: C, 67.76, H, 3.96; N, 17.55. $C_{27}H_{19}ClN_6O$ Calculated, %: C, 67.78, H, 3.97; N, 17.57.

5-(2-Benzyl-2*H*-tetrazol-5-yl)-4-(2-chlorophenyl)-3-methyl-6-phenylisoxazolo[5,4-*b*]pyridine (5k). Pale Yellow solid, mp: 238-240°C; IR spectrum, *v,* cm^{-1}: 1532 (N=N), 1652 (C=N); ^1H NMR spectrum (300 MHz, $CDCl_3$), δ, (ppm): 2.30 (s, 3H, isoxazole CH_3), 4.65 (s, 2H, N-CH_2),6.92-7.92 (m, 14H, Ar-H); ^{13}C NMR (75MHz, $CDCl_3$), δ, (ppm): 12.85, 60.70, 101.14, 120.58, 127.85, 128.21, 128.65, 130.45, 133.67, 134.60, 135.66, 136.65, 138.25, 139.67, 140.14, 141.31, 144.52, 146.25, 149.36, 150.27, 152.11, 155.25, 157.36, 160.03, 161.55, 168.61, 169.49. Mass spectrum: *m/z* 479 $[M + H]^+$. Found, %: C, 67.76, H, 3.95; N, 17.56. $C_{27}H_{19}ClN_6O$ Calculated, %: C, 67.78, H, 3.97; N, 17.57.

5-(2-Benzyl-2*H*-tetrazol-5-yl)-4-(4-methoxyphenyl)-3-methyl-6-phenylisoxazolo[5,4-*b*]pyridine (5l). Pale Yellow solid, mp 228-230°C; IR spectrum, *v,* cm^{-1}: 1536 (N=N), 1657 (C=N); ^1H NMR spectrum (300 MHz, $CDCl_3$), δ, (ppm): 2.28 (s, 3H, isoxazole CH_3), 3.68 (s, 3H, OCH_3), 4.65 (s, 2H, N-CH_2),6.88-7.93 (m, 14H, Ar-H); ^{13}C NMR (75MHz, $CDCl_3$), δ, (ppm): 12.92, 56.92, 61.36, 104.22, 121.93, 126.87, 128.47, 130.36, 133.91, 135.22, 136.55, 137.26, 139.32, 140.10, 142.92, 143.24, 145.31, 148.26, 149.67, 151.26, 152.91, 153.88, 156.98, 159.15, 160.39, 163.25, 170.16, 171.44; Mass spectrum: *m/z* 475 $[M + H]^+$. Found, %: C, 70.85, H, 4.62; N, 17.70. $C_{28}H_{22}N_6O_2$ Calculated, %: C, 70.88, H, 4.64; N, 17.72.

Biological activity

In vitro anticancer Activity

The human cell cultures HeLa (cervical), Ehrlich Ascites Carcinoma (EAC) & MCF-7 (breast cancer) cell lines were obtained from National Center for Cancer Cell Sciences (NCCS), Pune, India. These cell lines were grown in recommended media supplemented with 10% FBS, 1% L-glutamine and 1% penicillin streptomycine amphotericin B in a 5% CO_2 humidified atmosphere at 37°C. Cells were seeded in 25 cm^2 tissue culture flasks (Tarsons, India), at 250,000 cells/flask in a total volume of 9 mL. When confluent, all the cells were trypsinized (using Trypsin eEDTA, HiMedia, Mumbai, India), and seeded in 96-well plates (Tarsons, India). The cell suspension of 1 x 10^5 cells/mL was prepared in complete growth medium. Stock solutions of the compounds were prepared in DMSO. The stock solutions were serially diluted with complete growth medium containing 50 mg/ mL of gentamycin to obtain working test solution of required concentrations (having <1% DMSO). The 100 mL of cell suspension was added to each well of the 96-well plates. The test materials in complete growth medium (100 µL) were added after 24 h incubation to the wells containing cell suspension. After 48 h of treatment with different concentrations of test compounds, the cells were incubated with MTT (2.5 mg/mL) for 2 h. The medium was then removed and 100 µL of DMSO were added in to each well to dissolve formazan crystals, the metabolite of MTT. After thoroughly mixing, the plate was read at 490 nm for optical density that is directly correlated with cell quantity. The cytotoxic effects of the compounds were calculated as percentage inhibition in cell growth as per the formula. % cytotoxicity = 1 - [(O.D. in sample well)/(O.D. in control well)] x 100.

***In vivo* anticancer activity**

Adult female Swiss albino mice (Mahaveer Enterprises, Hyderabad, India) of 8 weeks old at study start (mean weight in the range of 20-25 g) were selected and housed in polypropylene cages in a room where the congenial temperature was 27 ± 1 ºC and 12 h light and dark cycles were maintained. The animals were allowed to acclimatize to the environment for 7 days and supplied with a standard pellet diet and water ad libitum. All procedures using animals were reviewed and approved by the Institutional Animal Ethical Committee of Kakatiya University. The animals were divided into seven groups (n = 10). The normal group was not inoculated with tumor cells, while six groups were injected with EAC cells (0.2 mL of 2 x 10^6 cells/mice) intraperitoneally. This was taken as day '0' and the experimental treatment started 24 h later. From the 1st day, 100 mL/mouse per day of sterile saline was administered intraperitoneally to the negative control group (EAC-bearing mice). Compounds and at doses of 5 mg/kg and 10 mg/kg respectively were administered each day to the treated groups and the standard drug *Cisplatin* at a dose of 5 mg/ kg was administered to each animal from the positive control group. The pharmacological treatment lasted for 9 days. Fourteen days after the treatment, five mice from each group were sacrificed for the study of antitumor activity. The rest of the animal group was kept to check the mean survival time of EAC tumor bearing hosts. The antitumor effects of the extracts were determined by the change in body weight, mean survival time (MST), and percentage increased life span (% ILS). The MST of each group containing five mice was identified by recording the mortality on a daily basis for 30 days, and the % ILS was calculated using the following equations. MST = (day of the first death + day of the last death)/2; ILS (%) = [(mean survival time of treated group/mean survival time of control group) - 1] x 100. The effect of compounds and was also assessed by the determination of the body weight, tumor volume, packed cell volume and viable tumor cell count of EAC-bearing mice by the Trypan blue incorporation method.

Anti-inflammatory activity

Anti-inflammatory activity was determined by carrageenan induced paw edema method. Wistar rats of either sex weighing 150-200 g were divided into 6 groups (n=6) and they were fasted 18 h before the experiment with water *ad libitum*. Group-I received 1% sodium CMC (negative control), Group-II received *Ibuprofen* at a dose of 100 mg/kg (positive control) and Group-III to VI were given the compounds **5a-l** (100 mg/kg). All the compounds **5a-l** were given in oral route. After 30 minutes, 0.1 mL of 1% carrageenan suspension in normal saline was injected into the subplantar region of the left hind paw of each rat to induce edema. The edema volumes of the injected paw measured with the help of plethysmograph at the interval of 0, 1, 2, 4 and 6 h. The difference between the paw volumes of treated animals were compared with that of the control group and the mean edema volume was calculated. Percentage inhibition was calculated as per the formula, %inhibition= [Vo-Vt)/Vo] x 100, where Vo = volume of the paw control at time t, Vt = volume of the paw of drug treated at time t.

In vitro cylcooxygenase inhibition studies: The compounds, **5a-l** were tested for their ability to inhibit *in vitro* COX-1 and COX-2 using a colorimetric COX (ovine) inhibitor screening kit (Catalog No. 760 112, Cayman Chemicals Inc., Ann Arbor, MI,USA) using the previously established method [36].

Molecular Docking

Molecular docking is used to find the exact binding conformation and orientation of the ligand molecule into the active site of the protein. The newly synthesized tetrazole-linked isoxazolo[5,4-*b*]pyridines **5a-l** and standard *Gefitinib* were docked against EGFR (Epidermal Growth Factor Receptor) kinase protein using SWISS AutoDock, an automated docking tool [37-38]. The docking process involves four main steps: (i) protein preparation, (ii) ligand preparation, (iii) grid preparation, and (iv) docking. The Lamarckian genetic algorithm has been used as the search algorithm to search for the best conformers. The initial population size was set randomly as 150 individuals and ten generation was set for each genetic algorithm run and the maximum number of energy evaluations was set to 2,500,000. The grid box size was centered at 8.671 Å × -8.036 Å × 0.67 Å and the dimensions of the grid box have been set as 40, 40, 40 (X, Y, Z coordinates) so as to include all the active site residues.

Conclusions

In conclusion, we reported a simple and efficient multi-component protocol for the synthesis of isoxazolo[5,4-*b*]pyridines using inexpensive starting materials. The title compounds were by [2+3] cyclo addition of isoxazolo[5,4-*b*]pyridines with aryl azides. The use of the catalyst in this reaction (basic alumina) has the advantage of enhanced yields and simple work-up compared to $FeCl_3$ alone. The newly synthesized compounds **5a-l** were evaluated for the *in vitro* and *in vivo* anticancer activity and anti inflammatory activities. Compounds **5c, 5d** is proved to prossess remarkable anticancer activity, and anti-inflammatory activity. By effecting a simple modification in structure, a new potent anolog can be generated with the desired activity with good efficacy. It also requires further studies to reveal structure-activity relationship.

Acknowledgements

The authors are grateful to the Director, CSIR-Indian Institute of Chemical Technology, Hyderabad, Telangana for recording spectra.

Consent For Publication

Not applicable

Availability Of Data And Materials

The data and supportive information are available within the article.

Conflict Of Interest

The authors declare no conflict of interest, financial or otherwise.

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