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SYNTHESIS AND ANTICANCER ACTIVITY OF SOME NOVEL THIENO-1,2,3-TRIAZINES

Saravanan Janardhanan* and Manju Bharghavi

Department of Pharmaceutical Chemistry, PES College of Pharmacy, Hanumanthanagar, Bangalore-50. Karnataka, India.

***Corresponding Author: Dr. Saravanan Janardhanan**
Department of Pharmaceutical Chemistry, PES College of Pharmacy, Hanumanthanagar, Bangalore-50. Karnataka, India.

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ABSTRACT
The main aim of this work was to synthesis a thieno-1,2,3- triazines by using Gewald reaction and its analogues were synthesized by diazotization; 3-N-p-tolyl-5,6-dimethyl thieno[2,3-d][1,2,3]-triazin-4-(3H)-one, *3-N-methyl-5,6-trimethylene thieno-[2,3-d][1,2,3]-triazin-4-(3H)-one*, 3-N-(4-chloro phenyl)–5,6-trimethylene thieno[2,3-d][1,2,3]-triazin-4-(3H)-one and 3-N-methyl–5,6-tetramethylene thieno[2,3-d][1,2,3]-triazin-4-(3H)-one. The synthesized thieno-1,2,3- triazines derivatives subjected to *in vitro* anticancer activity screening by using MTT assay in Ht29 colon cancer cell lines and Doxorubicin as standard. The prepared analogues were characterized by FT-IR, ^{1}H-NMR and mass spectra. The result reveals that most of the synthesized compounds showed anticancer properties.

KEYWORDS: Gewald reaction, Diazotization, Anticancer activity, Doxorubicin.

INTRODUCTION
Cancer, an important cause of mortality word wide next to cardiovascular disease, is characterized by the deregulation of signaling pathways with an initial loss of controlled cell division and growth, cell invasion, and finally, result in metastasis.[1] Colon cancer is a kind of digestive tract malignant tumor, commonly occurring at the junction of the rectum and sigmoid colon. The incidence of colon cancer begins to rise at age 40 and peaks at age 60 to 75, and the main life-style related cause is high-fat diet and inadequate cellulose intake. Colon cancer ranks third among the leading causes of cancer-associated death after lung and prostate cancer for men and after lung and breast cancer for women.[2] In recent years, there is an upward trend in India as the improvement of people's living standard and changes in diet. Accordingly, the development of novel chemotherapeutic agents is an urgent issue.

The triazine is a six-membered heterocyclic ring, analogous to the benzene ring but with three carbons replaced by nitrogens. 1,2,3-Triazines are a class of biologically active compounds that exhibit a broad spectrum of activities, including antibacterial, antifungal, antiviral, antiproliferative, analgesic and anti-inflammatory properties.[3] 1,2,3-triazine is the least explored one, till date. But, clinically 1,2,3-triazine derivatives are more acceptable because of potent efficacy and minimal side effect.[4]

Thiophene-containing compounds are also well known to exhibit various pharmacological effects; anti-HIV PR inhibitors,[5] anti-inflammatory,[6] anti-breast cancer,[7] anti-protozoal,[8] antitumor agents,[9] antimycobacterial activity,[10] inhibitors of EGF-RTK (epidermal growth factor receptor tyrosine kinase),[11] anti-hypotensive[12] and anti-convulsant.[13]

In light of this we planned to synthesize a series of new 1,2,3-triazines carrying thiophene moieties in the hope of obtaining new products of superior biological activity such as anticancer activity.

MATERIALS AND METHODS
All melting points were taken on an Electrothermal IA 9100 series digital melting point apparatus. IR (KBr) were recorded on Perkin-Elmer FT-IR RX-II, H^{1} NMR spectra were recorded on brucker AMX 400. Elemental analyses were within ± 0.4% of their calculated values.

Synthesis of the compounds
2-amino-3-N-substituted carboxamido-4,5-disubstituted thiophenes (ADVJS-3 a – 3 m)
A mixture of required active methylenic ketone, substituted cyano acetanilide, ammonium acetate and glacial acetic acid (2ml) in benzene (80ml) was refluxed for 10hrs in a Dean stark apparatus with the arrangement of water separation. The reaction mixture was cooled, diluted with benzene and washed successively with water, 10% sodium carbonate solution and dried over

anhydrous sodium sulphate. The solvent was removed under vacuum. Later, the crude intermediate was stirred with sulphur in ethanol with the addition of diethylamine drop wise for 1 hr at 45-50^{0}C, chilled overnight and the solid obtained was filtered washed with ethanol to yield yellow crystalline products (ADVJS-3 a – 3 m).

Reaction

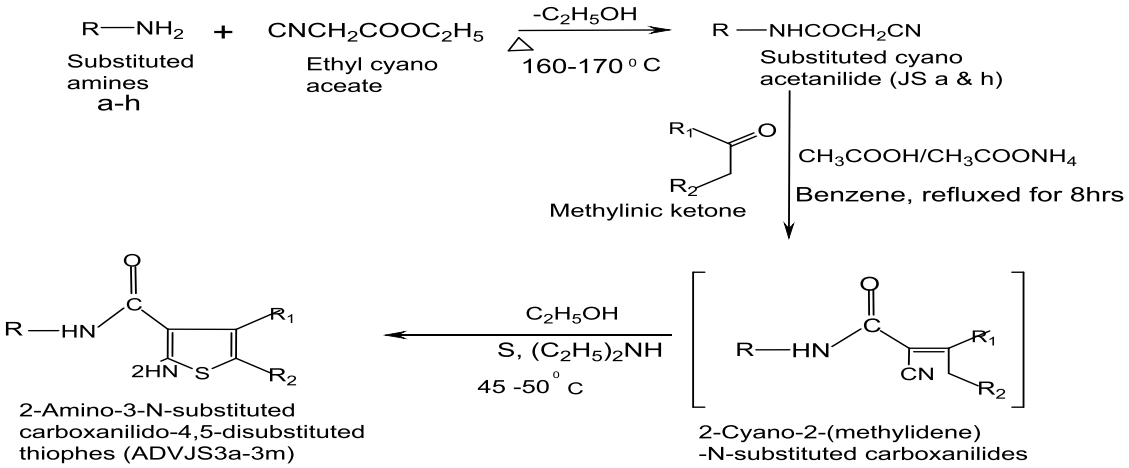

Figure No. 1: Synthesis of 2- amino-3-N- substituted carboxanilido -4,5- disubstituted thiophenes (ADVJS 3a-3m).

Where: R = -H, methyl, phenyl, o- chloro phenyl, m- chloro phenyl, p- chloro phenyl
o-tolyl, m-tolyl, p-tolyl, 3-chloro-4-fluoro phenyl
R_1, R_2 = -CH_3, - $(CH_2)_3$, - $(CH_2)_4$, - $(CH_2)_5$

Syntheses of bicyclic/tricyclic thieno-1,2,3-triazin-4-ones (ADVJS- 4 a -4 m)

A mixture of the appropriate parent compound (ADVJS-3 a – 3 m) in 30 ml of glacial acetic acid was warmed until the compound was dissolved. Cooled the mixture to room temperature, 20ml of Conc. HCl was added and cooled to a temp. below 5^{0}C . Then to the above mixture an ice cold solution of $NaNO_2$ (0.03mole) in water (25ml) was added drop wise with constant stirring. Temperature was maintained below 5^{0}C. The product obtained was filtered, dried and washed with methanol to get the desired pure thieno-1,2,3-triazin-4-ones.

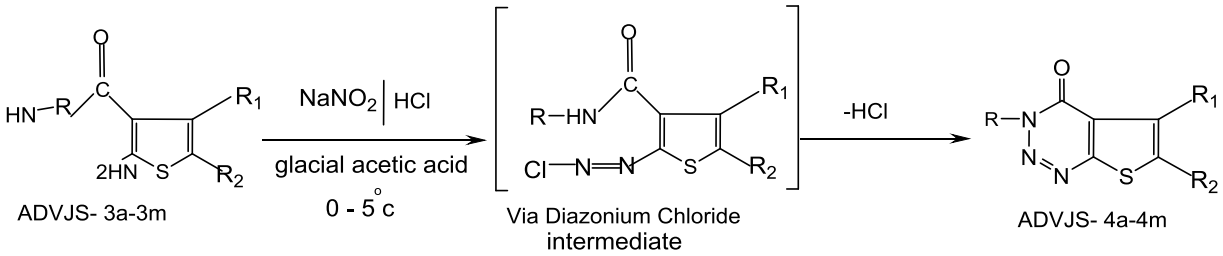

Figure No. 2: Synthesis of new bicyclic/tricyclic thieno- 1,2,3 – triazine – 4 – ones (ADVJS- 4 a -4 m).

Where: R = -H, methyl, phenyl, o- chloro phenyl, m- chloro phenyl, p- chloro phenyl
o-tolyl, m-tolyl, p-tolyl, 3-chloro-4-fluoro phenyl
R_1, R_2 = -CH_3, - $(CH_2)_3$, - $(CH_2)_4$, - $(CH_2)_5$

ADVJS 4a 3-N-p-tolyl -5,6-dimethyl thieno[2,3-d][1,2,3]-triazin-4-(3H)-one

IR max cm $^{-1}$ = Ar-CH 3035.23: -Ali-CH 2982.54; Arom C=C 1498. 63; - CO –1692; (C-N)-759.23. ^{1}H NMR: (Solvent –$CDCl_3$) 7.6 (t,2H, Arom)-7.3 (t,2H, Arom), 2.61(s,4H, methyl proton), 2.5 (t, 2H, -CH_2-);, 1.9 (m,2H, methyl protons). MS (%) 290.06 (M^+ 100.0%), 270.07(15.3%), 271.06 (1.9%), 271.07 (1.4%) 3-N-p-tolyl -5,6-dimethyl thieno[2,3-d][1,2,3]-triazin-4-(3H)-one, *3-N-methyl-5,6-trimethylene thieno-[2,3-d][1,2,3]-triazin-4-(3H)-one,* 3-N-(4-chloro phenyl)–5,6-trimethylene thieno[2,3-d][1,2,3]-triazin-4-(3H)-one, 3-N-methyl–5,6-tetramethylene thieno[2,3-d][1,2,3]-triazin-4-(3H)-one.

ADVJS 4d *3-N-methyl-5,6-trimethylene thieno-[2,3-d][1,2,3]-triazin-4-(3H)-one*

IR max cm $^{-1}$ = Ar-CH 3024.83: -Ali-CH 2978.54; Arom C=C 1506.46; - CO –1660; (C-N)-746.23. ^{1}H NMR: (Solvent-$CDCl_3$): 2.7 (s, 3H, - CH_3), 2.6 (m, 4H, methylenic protons), 1.9 (m, 2H, methylenic protons). MS (%) 195.05 (M^+ 100.0%), 128.05 (10.7%), 109.04 (4.5%).

ADVJS 4f 3-N-(4-chloro phenyl)–5,6-trimethylene thieno[2,3-d][1,2,3]-triazin-4-(3H)-one

IR max cm^{-1} =Ar-CH 3098.35: -Ali-CH 2968.57; Arom C=C 1508.24; - CO –1688; (C-N) -773.42. ^1H NMR: Solvent – $CDCl_3$ 7.2(d,2H, Arom), 7.8 (d,2H, Arom), 2.8 (m,4H, methylenic protons), 1.6 (m,2H, methylenic protons). MS (%) 268.04 (M+100.0%), 219.04(32.5%), 181.04 (18.6%).

ADVJS 4g 3-N-methyl–5,6-tetramethylene thieno[2,3-d][1,2,3]-triazin-4-(3H)-one

IR max cm^{-1} = Ar-CH 3044.93: -Ali-CH 2967.98; Arom C=C 1502.37; - CO –1710; (C-N) -739.25. ^1H NMR: Solvent – $CDCl_3$ 2.6 (m,4H, methylenic proton), 2.3 (s, 3H CH_3), 2.1 (m,4H, methylenic protons). MS (%) 221.08 (M+ 100.0%), 184.08 (17.2%), 136.07 (4.5%).

ADVJS 4 k *3*-N-(phenyl)-5,6-tetramethylene thieno[2,3-d][1,2,3]-triazin-4-(3H)-one

IR max cm^{-1} = Ar-CH 3013.34: -Ali-CH 2963.25; Arom C=C 1507.26; - CO –1684; (C-N) -742.25. ^1H NMR: Solvent – $CDCl_3$ 7.6 (t,2H, Arom),7.3 (t,2H, Arom), 7.2 (s,1H, Arom) 2.6 (m,4H, methylenic proton),), 2.1 (m,4H, methylenic protons),1.9 (m,2H, methylenic protons).

MS (%) 297.09 (M+ 100.0%), 274.09 (17.8%), 255.10 (4.9%)

ANTICANCER ACTIVITY

Procurement of cancer cell lines

The HT-29 (human colorectal adenocarcinoma) cell line was initially procured from the National Centre for Cell Sciences (NCCS), Pune, India, and maintained in DMEM. The cell line was cultured in 25 cm^2 tissue culture flask with DMEM supplemented with 10% FBS, L-glutamine, sodium bicarbonate and antibiotic solution containing: penicillin (100 U/ml), streptomycin (100 µg/ml). Cultured cell line was kept at 37°C in a humidified 5% CO_2 incubator (VWR, USA). Two-day-old confluent monolayer of cells were trypsinized and the cells were suspended in 10% growth medium, 100 µl cell suspension (5×10^4 cells/well) was seeded in 96-well tissue culture plate and incubated at 37°C in a humidified 5% CO_2 incubator for 24 h. The viability of cells was evaluated by direct observation of cells by Inverted phase contrast microscope and followed by MTT assay method.

MTT Viability assay[14]

The cytotoxic potential of the most active derivatives (as mentioned in the above section) was evaluated by MTT (3,4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide) assay which is based on the reduction of the yellow colored water-soluble tetrazolium dye MTT by the mitochondrial lactate dehydrogenase formed by the live cells to the formazan crystals, which display purple color upon dissolution into the suitable solvent. The intensity of the purple color is directly proportional to the number of viable cells and can be measured by spectrophotometer at 570 nm. The HT-29 cells were treated with different concentrations of these derivatives (12.5, 25, 50 and 100 µg/ml) for 24 h and observed for cytotoxicity by MTT assay using ELISA reader. Anticancer efficacy of selected synthesized compounds was measured in terms of percentage of growth inhibition by using following formula:

% of inhibition = [Absorbance of sample/ Absorbance of control] X 100

RESULTS AND DISCUSSION

Synthesis

The synthesis of Syntheses of Synthesis of 2- amino-3-N- substituted carboxanilido -4,5- disubstituted thiophenes (ADVJS 3a-3m) and bicyclic/tricyclic thieno-1,2,3-triazin-4-ones(ADVJS -4a -4m) is shown in [Figure 1 and 2]. All synthesized compounds subjected for physical melting point determination data shown in [Table 1] and the structure of selected synthesized compounds was confirmed by IR, ^1H NMR and MS spectral data.

Anti-cancer activity

The anticancer potential of the most active derivatives (as mentioned in the above section) was evaluated by MTT (3,4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide) assay which is based on the reduction of the yellow colored water-soluble tetrazolium dye MTT by the mitochondrial lactate dehydrogenase formed by the live cells to the formazan crystals, which display purple color upon dissolution into the suitable solvent. The intensity of the purple color is directly proportional to the number of viable cells and can be measured by spectrophotometer at 570 nm. The HT-29 cells were treated with different concentrations of these derivatives (12.5, 25, 50, 100 µg/ml) for 24 h and observed for anticancer activity by MTT assay using ELISA reader. Doxorubicin was used as standard. The cell survival plots were drawn between the % viability, and different concentrations of these test derivatives have been given in Table No.2. The observations in statistical data of cell anticancer study suggest that the different test derivatives have decreased the cell viability in a dose-dependent manner. The % of cell viability decreased from 99 to 10% on treatment with the different concentrations of these test derivatives. Selected new synthesized thienotriazines, ADVJS -4a to 4d R shows decrease in the cell viability followed by ADVJS -4e to 4h and ADVJS -4i to 4 l. it was also particularly noticed that the three compounds; ADVJS -4g, ADVJS -4h and ADVJS -4i possessing cyclohexyl group at R1, R2 positions exhibited anticancer activity better than the other compounds of the series (Table No. 2).

Table No. 1: Physical data of the new bicyclic/tricyclic Thieno 1,2,3 – triazine – 4 – ones (ADVJS- 4 a-4m).

Sl. No.	Synthesized Compound	R	R_1,R_2	M.P.(^OC)	
1	ADVJS- 4 a	p-tolyl,	$-CH_3$	152	**Triazines were Washed only With methanol to get pure compounds**
2	ADVJS- 4 b	p- chloro phenyl	$-CH_3$	166	
3	ADVJS- 4 c	3-chloro-4-fluoro phenyl	$-CH_3$	178	
4	ADVJS- 4 d	methyl	$-(CH_2)_3$	134	
5	ADVJS- 4 e	o-tolyl,	$-(CH_2)_3$	148	
6	ADVJS- 4 f	p- chloro phenyl	$-(CH_2)_3$	167	
7	ADVJS- 4 g	methyl	$-(CH_2)_4-$	141	
8	ADVJS- 4 h	o-tolyl	$-(CH_2)_4-$	152	
9	ADVJS- 4 i	m-tolyl,	$-(CH_2)_4-$	118	
10	ADVJS- 4 j	H	$-(CH_2)_5-$	124	
11	ADVJS- 4 k	phenyl	$-(CH_2)_5-$	151	
12	ADVJS- 4 l	o-tolyl	$-(CH_2)_5-$	158	
13	ADVJS- 4 m	m- chloro phenyl	$--(CH_2)_5-$	165	

Table No. 2: Anticancer activity synthesized thieno-1,2,3-triazines derivatives.

SAMPLE CODE	CONCENTRATION(µg/ml)	ABSORBANCE (540 nm)	PERCENTAGE OF VIABILITY
Control		0.6495	
ADVJS- 4 a	12.5	0.4886	75.22±0.78
	25	0.4572	70.39±0.97
	50	0.3956	60.90±0.393
	100	0.312	48.03±0.156
ADVJS- 4 b	12.5	0.4943	76.10±0.596
	25	0.4017	61.84±0.511
	50	0.3602	55.45±0.466
	100	0.3193	49.16±0.438
ADVJS- 4 c	12.5	0.4989	76.81±0.332
	25	0.4836	74.45±0.485
	50	0.4043	62.24±0.831
	100	0.3035	46.72±0.256
ADVJS- 4 d	12.5	0.4235	65.20±0.031
	25	0.3569	54.94±0.615
	50	0.3083	47.46±0.825
	100	0.2393	36.84±0.259
ADVJS- 4 e	12.5	0.4137	63.69±0.01
	25	0.3178	48.92±0.61
	50	0.30854	47.50±0.54
	100	0.2863	44.08±0.16
ADVJS- 4 f	12.5	0.4411	67.91±0.98
	25	0.4308	66.32±0.46
	50	0.4217	59.01±0.66
	100	0.3833	43.40±0.74
ADVJS- 4 g	12.5	0.4525	69.68±0.61
	25	0.4232	65.15±0.37
	50	0.3695	56.88±0.53
	100	0.3601	44.59±0.24
ADVJS- 4 h	12.5	0.4117	63.38±0.09
	25	0.3886	59.83±0.39
	50	0.3787	58.30±0.95
	100	0.3038	46.77±0.19
ADVJS- 4 i	12.5	0.3412	52.53±0.75
	25	0.3064	47.17±0.98
	50	0.2819	43.40±0.74
	100	0.2692	41.44±0.71
ADVJS- 4 j	12.5	0.3867	59.53±0.62
	25	0.3401	52.36±0.64
	50	0.2948	45.38±0.06

	100	0.2877	44.29±0.12
ADVJS- 4 l	12.5	0.2984	45.93±0.31
	25	0.2878	44.31±0.85
	50	0.2687	41.37±0.48
	100	0.2597	39.98±0.35
ADVJS- 4 m	12.5	0.3687	59.52±0.62
	25	0.3410	52.46±0.64
	50	0.2489	46.28±0.06
	100	0.2787	43.19±0.12

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