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The biological activity of new thieno[2,3-c]pyrazole compounds as anti-oxidants against toxicity of 4-nonylphenol in *Clarias gariepinus*

Alaa El-Din H. Sayed [a,*], Remon M. Zaki [b], Adel M. Kamal El-Dean [b], Abdullah Y. Abdulrazzaq [b]

[a] *Zoology Department, Faculty of Science, Assiut University, Assiut 71516, Egypt*
[b] *Chemistry Department, Faculty of Science, Assiut University, Assiut 71516, Egypt*

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ABSTRACT

Synthesis of bi functionally substituted thieno[2,3-c]pyrazole compounds was carried out by a new method. The substituted group at position five is namely (carbonitrile, carboxamide, *N*-(substitutedphenyl) carboxamide and benzoyl group). Chloroacetylation of the amino thieno[2,3-c]pyrazolecarboxamide compound afforded the chloroacetyl amino derivative. The chemical structure of the newly synthesized compounds was established by elemental and spectral analysis including IR, ^{1}H NMR spectra in addition to ^{13}C NMR and mass spectra for most of them. In the present work, we assessed the role of the new synthesized thieno[2,3-c]pyrazole compounds as antioxidants against the toxicity of the 4-nonylphenol on the red blood cells of the most economically important Nile fishes namely African catfish (*Clarias gariepinus*). The erythrocytes alterations were used as biological indicators to detect those effects. After exposure to 4-nonylphenol, the erythrocytes malformations (swelled cells, sickle cells, tear drop like cells, acanthocytes, and vacuolated cells) were recorded in highest number in comparison with other groups control and those injected with thieno[2,3-c]pyrazole compounds. So, the new thieno[2,3-c]pyrazole compounds can be used as antioxidants against toxicity of 4-nonylphenol on fishes.

1. Introduction

The Pyrazoles and condensed pyrazoles are very important class of heterocyclic compounds and are considered very biologically important compounds. Pyrazole derivatives revealed many biological activities, such as anti-inflammatory [1–5], antimicrobial [6,7], antioxidant [8], anticancer [9–11], fungicidal [10], and antiviral activities [10,12,13]. Some thienopyrazole of type A is used for inhibiting PDE 7 (Phosphodiesterase 7) selectively which is responsible for allergy, immunological diseases and inflammatory diseases [14]. Bindi et al. [15] reported a series of thienopyrazoles to demonstrate their activity as a potent inhibitor for aurora kinase. In continuation of our previous work in the synthesis of heterocyclic compounds containing pyrazolemoiety [16–23,24–29]. Herein, we reported a novel facile method for synthesis of thieno[2,3-c]pyrazole substituted at position four and five like structure **A**. The difficulty of synthesis bi- functionally substituted thieno[2,3-c]pyrazole like structure **A** is attributed to the most synthesis based on starting with the moiety containing mercapto group adjacent to cyano group. In the case of chloropyrazolecarbonitrile compound (**4**), the chlorine atom is very difficult to be displaced by sulfur atom. So, we tried to displace the chlorine atom with sulfur using thiourea as reported in other moieties, but all attempts, failed.

After several attempts, we displaced the chlorine atom with sulfur atom in the presence of sodium borohydride, and using the product in situ without isolation to be subjected in the next reaction with α-halogenated alkylating agents to afford compounds **6a–f**, which underwent Thorpe–Ziegler cyclization to give 4-amino-3-methyl-1-phenyl-5-substituted-1*H*-thieno[2,3-c] pyrazole (**7a–f**) [30].

4-Nonylphenol is persistent in the environment as one of the breakdown products of nonylphenols in aquatic organisms [31–33]. The negative effects of 4-nonylphenol has been studied in fish especially on the catfish *Clarias gariepinus* as toxicological model [32,33–37]. The hemotoxic effect of the 4-nonylphenol has been reported indicating the adverse damage at 0.1 mg/l on *C. gariepinus* [32]. Blood is a good indicator to determine the functional status of exposed animals to toxicants [32,38].

Also, the blood cell's alterations have been used as bio-indicators for the study of the effects of toxicants and stressors on fish

* Corresponding author. Fax: +20 882342708.
E-mail address: alaa_h254@yahoo.com (A.H. Sayed).

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Table 1
The physical properties of compounds (6a–f) and (7a–f).

Compound no.	(6a–f) and (7a–f)			Elemental analysis Calcd/found			
	Mol. formula	M.P.	Solvent of crystallizationYield%	C	H	N	S
6a	$C_{13}H_{10}N_4S$	78	Ethanol	61.40	3.96	22.03	12.61
	254.32	–80	85	61.15	4.10	21.93	12.75
6b	$C_{13}H_{12}N_4OS$	144	Ethanol	57.34	4.44	20.57	11.77
	272.33	–146	80	57.26	4.50	20.60	11.60
6c	$C_{19}H_{16}N_4OS$	128	Ethanol	65.50	4.63	16.08	9.20
	348.43	–130	68.75	65.72	4.55	15.95	9.40
6d	$C_{20}H_{18}N_4OS$	118	Ethanol	66.28	5.01	15.46	8.85
	362.46	–120	63	66.08	4.95	15.35	8.68
6e	$C_{20}H_{18}N_4O_2S$	148	Ethanol	63.47	4.79	14.80	8.47
	378.46	–150	74	63.55	4.70	14.77	8.32
6f	$C_{19}H_{15}ClN_4OS$	132	Ethanol	59.60	3.95	14.63	8.37
	382.87	–134	74	59.80	3.90	14.70	8.50
7a	$C_{13}H_{10}N_4S$	198	Ethanol +Dioxane	61.40	3.96	22.03	12.61
	254.32	–200	75	61.30	3.90	22.16	12.52
7b	$C_{13}H_{12}N_4OS$	214	Ethanol +Dioxane	57.34	4.44	20.57	11.77
	272.33	–216	70	57.30	4.40	20.55	11.67
7c	$C_{19}H_{16}N_4OS$	202	Ethanol +Dioxane	65.50	4.63	16.08	9.20
	348.43	–204	68	65.76	4.50	16.15	9.35
7d	$C_{20}H_{18}N_4OS$	200	Ethanol +Dioxane	66.28	5.01	15.46	8.85
	362.46	–202	70	66.10	4.90	15.50	8.75
7e	$C_{20}H_{18}N_4O_2S$	208	Ethanol +Dioxane	63.47	4.79	14.80	8.47
	378.46	–210	65	63.42	4.85	14.60	8.55
7f	$C_{19}H_{15}ClN_4OS$	204	Ethanol +Dioxane	59.60	3.95	14.63	8.37
	382.87	–206	70	59.70	4.00	14.57	8.41

[32,35,39] and any changes therefore reflected in their morphology and distribution in the blood [40]. The previous studies have been reported that the damage which was carried out to the blood and heamapoietic organs in fish may be associated due to either change in environmental conditions or water born pollutants [41–43]. It was stated by many authors that various abnormal morphological forms of erythrocytes are effective indicators of cytotoxicity [32,44]. African catfish (*C. gariepinus*) has been used in fundamental research and considered an excellent model for toxicological studies [45–47], since it has a well-documented biology [47,48].

The present work was, therefore, undertaken to examine the ability of the newly synthesized thieno[2,3-c]pyrazole compounds to prevent or protect the toxic effects of 4-nonylphenol on erythrocytes of the catfish, *C. gariepinus*.

2. Materials and methods

2.1. Chemical experimental

All melting points are uncorrected and measured on a Fisher-John apparatus. IR spectra were recorded (KBr) with a PerkinElmer 1430 Spectrophotometer. ^{1}H NMR and ^{13}C NMR spectra were obtained on a Varian EM-390 MHz (90 MHz) and Bruker 400 MHz spectrometers in $CDCl_3$, DMSO-d_6 and CF_3CO_2D using Me_4Si as internal standard, and chemical shifts are expressed as ppm. Mass spectra were measured on a Joel-JMS 600 spectrometer. Analytical data were obtained on Elemental Analyze system GmbH-VarioEL V.3 microanalyzer in the central lab of Assiut University. The progress of the reaction and the purity of products were monitored by thin layer chromatography (TLC). The substituted pyrazole compounds **1–4** were synthesized according to literature procedure [29], with m.p. 124–126 °C for compound **1**, 140–142 °C for compound **2**, 132–134 °C for compound **3** and 118–120 °C for compound **4** respectively.

2.1.1. 3-Methyl-1-phenyl-5-substitutedthiopyrazole-4-carbonitrile (6a–f)

2.1.1.1. General procedure (1). A suspension of finally powered sulfur metal (4 g, 0.125 mol) in absolute ethanol (60 ml) in an ice bath, the sodium borohydride (4 g, 0.105 mol) was added in small portions until all sulfur powder dissolved, then the chlorocyanopyrazole **4** (10 g, 46 mmol) was added to the reaction mixture with stirring in an ice bath for one hour. The reaction mixture was refluxed for four hours followed by cooling, and then the α-halogenated alkylating agent (46 mmol) was added to the mixture. The reaction mixture was left overnight with stirring. The solid precipitate, which formed on cold was filtered off, dried and recrystallized from the proper solvent as white crystals. The physical properties and spectral data of compounds **6 a–f** are listed in Tables 3 and 4.

*2.1.2. 4-Amino-3-methyl-5-substituted-1-phenyl-1*H*-thieno[2,3-c]pyrazole-5-carbonitrile (7a–f)*

2.1.2.1. General procedure (2). A mixture of substituted pyrazole-carbonitrile compounds **6 a–f** (4 g, 16 mmol) in absolute ethanol (20 ml) and sodium ethoxide solution (2.5 ml) was gently refluxed for 10 minutes. The solid precipitate, which formed during reflux, was filtered off, dried and recrystallized from ethanol: dioxane 2:1 mixture as white needless. The physical properties and spectral data of compounds **7 a–f** are listed in Tables 1 and 2 .

*2.1.3. 4-(2-Chloroacetylamino)-3-methyl-1-phenyl-1*H*-thieno[2,3-c]pyrazole-5-carboxamide (8)*

A solution of amino carboxamide compound **7b** (2 g, 7 mmol) and chloroacetyl chloride (0.80 ml, 9 mmol) in dioxane (30 ml) was hated on water bath for 3 h. The solid product which obtained by cooling and pouring on diluted sodium carbonate solution was filtered off, dried and recrystallized from ethanol into smoke white crystals. Yield 2.10 g (86%); mp: 258–260 °C. IR spectrum: 3380, 3300, 3180 (NH, NH_2), 3050 (CH aromatic), 2920, 2850 (CH aliphatic), 1690, 1650 (2C = O amide). ^{1}H NMR (DMSO-d_6): 2.40 (s, 3H, CH_3), 4.50 (s, 2H, CH_2), 7.30–7.80 (m, 7H, ArH, NH_2), 10.50 (s, 1H, NH). ^{13}CNMR (100 MHz, DMSO-d_6): 13.4 (CH_3pyrazole), 42.8 (CH_2Cl), 120.0, 127.4, 130.1, 142.6, 148.2 (C), 117.1, 117.6, 124.8,

Table 2
Spectral analysis of compounds 6a–f and 7a–f.

Compound No.	IR (KBr, cm^{-1})	^1H NMR, ^{13}C NMR (ppm)	Mass spectra
6a	ν: 3035 (CH aromatic), 2985–2925 (CH aliphatic), 2275, 2227 (2CN) cm^{-1}	^1H NMR: (90 MHz, DMSO-d_6) δ:2.85 (s, 3H, CH_3), 3.85 (s, 2H, CH_2), 7.60–7.40 (m, 5H, ArH) ppm	EI-MS (*m*/*z*) (%) 254.40 (M^+)
6b	ν: 3450, 3300 (NH_2), 3050 (CH aromatic), 2920, 2850 (CH aliphatic), 2210 (CN), 1660 (CO amide), 1590 (C = N) cm^{-1}	^1H NMR: (400 MHz, DMSO-d_6) δ: 2.35 (s, 3H, CH_3), 3.30 (s, 2H, CH_2), 7.15 (s, 2H, NH_2 disappeared by D2O), 7.30–7.70 (m, 5H, ArH) ppm	EI-MS (*m*/*z*) (%) = 272.14 (M^+)
6c	ν: 3160 (NH), 3057 (CH aromatic), 2920.81,2885 (CH aliphatic), 2227 (CN), 1654 (CO) cm^{-1}	^1H NMR: (400 MHz, DMSO-d_6) δ: 2.56 (s, 3H, CH_3), 4.03 (s, 2H, CH_2), 7.08–7.52 (m, 10H, ArH), 10.15 (s,H,NH) ppm.	
6d	ν: 3130 (NH), 3070 (CH aromatic), 2905, 2885 (CH aliphatic), 2220 (CN), 1660 (CO) cm^{-1}	^1H NMR: (400 MHz, DMSO-d_6) δ: 2.31 (s, 3H, CH3 *p*-tolyl), 2.56(s, 3H, CH_3pyrazole), 4.03 (s, 2H, CH_2), 7.21–7.52 (m, 9H, ArH), 9.21(s,H,NH) ppm	
6e	ν: 3303 (NH), 3035 (CH aromatic), 2975, 2870 (CH aliphatic), 2229 (CN), 1654 (CO) cm^{-1}	^1H NMR: (400 MHz, DMSO-d_6) δ: 2.52 (s, 3H, CH_3pyrazole), 3.75 (s,3H, CH_3*P*-anisyl), 3.70 (s, 2H, CH_2), 7.30–8.00 (m, 9H, ArH), 9.15(s,H,NH) ppm.	
6f	ν: 3303 (NH), 3035 (CH aromatic), 2975, 2870 (CH aliphatic), 2229 (CN), 1654 (CO) cm^{-1}	^1H NMR: (400 MHz, DMSO-d_6) δ: 2.32 (s, 3H, CH3), 3.70 (s, 2H, CH_2), 7.33–7.99 (m, 9H, ArH), 10.28 (s,H,NH) ppm.	
7a	ν: 3455, 3359, 3200 (NH_2), 3045 (CH aromatic), 2950, 2890 (CH aliphatic), 2184 (CN) cm^{-1}	^1H NMR: (400 MHz, DMSO-d_6) δ: 3.37 (s, 3H, CH_3), 6.93 (s, 2H, NH_2), 7.26-7.56 (m, 5H, ArH) ppm. ^{13}C NMR (100 MHz, DMSO-d_6) δ: 13.0 (CH_3pyrazole), 71.22, 116.32, 140.79, 143.72, 147.79 (C), 117.16 (CN), 121.57, 125.98, 129.95, 138.31 (Phpyrazole) ppm.	ESI-MS: ($C_{13}H_{10}N_4S$), 254.33 (M^+)
7b	ν: 3400, 3305, 3190 ($2NH_2$), 3050 (CH aromatic), 2910(CH aliphatic) cm^{-1}, 1635 (CO amide), 1580 (C = N) cm^{-1}	^1H NMR: (90 MHz, DMSO-d_6) δ: 2.60 (s, 3H, CH_3), 6.90 (s, 2H, NH_2 amide), 7.0 (s, 2H, NH_2), 7.30-7.70 (m, 5H, ArH) ppm. ^{13}C NMR (100 MHz, DMSO-d_6) δ: 15.2 (CH_3pyrazole), 109.3, 121.5, 145.4, 145.4, 149.9 (C), 124.4, 128.2, 129.8, 133.1 (Phpyrazole), 169.8 ($CONH_2$).	
7c	ν: 3304, 3200 (NH_2), 3135 (NH), 3050 (CH aromatic), 2006, 2887 (CH aliphatic), 1685 (CO) cm^{-1}	^1H NMR: (400 MHz, DMSO-d_6) δ: 2.63 (s, 3H, CH_3), 6.64 (s, 2H, NH_2), 7.10–8.03 (m, 9H, ArH), 8.89 (s, H, NH) ppm	
7d	ν: 3289, 3215 (NH_2), 3130 (NH), 3070 (CH aromatic), 2905, 2885 (CH aliphatic), 1655 (CO) cm^{-1}	^1H NMR: (400 MHz, DMSO-d_6) δ: 2.30 (s, 3H, CH_3 p-tolyl), 2.63(s,3H,CH_3pyrazole) 6.64 (s, 2H, NH_2), 7.30-7.58 (m, 9H, ArH), 8.84 (s, H, NH) ppm	
7e	ν: 3429, 3328, 3274.74 ($2NH_2$, NH), 3030 (CH aromatic), 2860(CH aliphatic), 1624 (CO) cm^{-1}	^1H NMR: (400 MHz, DMSO-d_6) δ: 2.48 (s, 3H, CH_3pyrazole), 3.71 (s, 3H, CH_3*p*-anisyl), 7.15 (s, 2H, NH_2), 7.30–7.70 (m, 9H, ArH), 9.15 (s, H, NH) ppm	
7f	ν: 3429, 3336, 3275 ($2NH_2$, NH), 3050 (CH aromatic), 2880(CH aliphatic), 1640 (CO) cm^{-1}	^1H NMR: (400 MHz, DMSO-d_6) δ: 2.49 (s, 3H, CH_3), 7.25 (s, 2H, NH·), 7.31–7.71 (m, 9H, ArH), 9.37 (s, H, NH) ppm. ^{13}C NMR (100 MHz, DMSO-d_6) δ: 13.1 (CH_3pyrazole), 71.4, 116.3, 147.8, 156.0 (C), 121.0, 129.34, 132.7, 136.0 (*p*-ClPh), 123.1, 128.2, 130.8, 135.0 (Phpyrazole), 182.0 (CONH) ppm.	

125.7, 126.4, 138.4 (Phpyrazole), 158.0 (CONH), 166.4 ($CONH_2$). Found: C, 51.52; H, 4.90; Cl, 10.00; N, 15.95; S, 9.0%. Calcd.: $C_{15}H_{13}ClN_4O_2S$ (348.81): C, 51.65; H, 3.76; Cl, 10.16; N, 16.06; S, 9.12%.

2.2. Fish

Adult African catfish (*C. gariepinus*) were collected from the Assiut university Fish farm July 2013 and acclimated in aerated recirculating tank containing experimental media water for two months. Fish were fed with a commercial fish food twice a day and kept at approximately 28 °C with 12 h: 12 h light: dark cycle. In each closed re-circulating systems six fish were forked. During the acclimation period 20% of the water in each recirculating system was replaced daily and were fed 5% body weight twice a day with commercial pellets. Fish measuring the average size about 36.7 ± 1.38 cm in length and the average weight about 424.4 ± 27.34 g.

2.3. 4-Nonylphenol

4-Nonylphenol was obtained from Sigma–Aldrich (Schnelldrof, Germany) with purity 99.3%.

2.4. Experimental design

Prior to experiments, the fish were determined to be free of external parasites [49]. To test the biological effects of those chemicals compounds seven groups of adult fish (3 per group) were maintained in 100 L glass aquaria; one group as control, the second exposed to 0.1 mg/l 4-nonylphenol, and the other five groups were injected with different tested chemicals compounds as described in Table 3 for two weeks in triplicates for each group. The doses selected for this study were based on previous work [32]. During the experimental period, fish were fed 5% body weight twice a day with commercial pellets and the experimental media water was changed every day.

2.5. Erythrocytes alterations

Blood smears were obtained by caudal incision and blood was collected onto clean grease-free microscope slides after two weeks. The smears (seven slides/fish) were dried, fixed in absolute methanol for 10 min, and stained with Giemsa stain as reported previously [50] Slides were selected on the basis of staining quality, coded, randomized and scored blindly. In each group 10,000 cells (minimum of 1000 per slide) were examined using the method of [51] under a 40× objective and 10× eyepiece to identify morphologically altered erythrocytes in separate studies.

2.6. Statistical analysis

The means, standard divisions and ranges were estimated. One-way analysis of variance was used to analyze the data using SPSS software [52] at the 0.05 significance level. Tukey's HSD test was

Table 3
The fish groups exposed to 4-nonyphenol (0.1 mg/l), chemicals compounds (10 mg/kg body weight) and their combinations.

Group Treatment	1	2	3	4	5	6	7
4-Nonylphenol	0	0.1	0.1	0.1	0.1	0.1	0.1
4-Amino-3-methyl-1-phenyl-1*H*-thieno[2,3-c]pyrazole-5-carbonitrile **(7a)**	0	0	10	0	0	0	0
4-Amino-3-methyl-1-phenyl-1*H*-thieno[2,3-c]pyrazole-5-carboxamide **(7b)**	0	0	0	10	0	0	0
4-Amino-N-(4-methoxyphenyl)-3-methyl-1-phenyl-1*H*-thieno[2,3-c]pyrazole-5-carboxamide **(7e)**	0	0	0	0	0	10	0
4-Amino-N-(4-chlorophenyl)-3-methyl-1-phenyl-1*H*-thieno[2,3-c]pyrazole-5-carboxamide **(7f)**	0	0	0	0	10	0	0
4-(2-Chloroacetamido)-3-methyl-1-phenyl-1*H*-thieno[2,3-c]pyrazole-5-carboxamide **(8)**	0	0	0	0	0	0	10

used for multiple comparisons and to verify the frequency of erythrocyte alterations.

2.7. Ethics statement

All experiments were carried out in accordance with the Egyptian laws and University guidelines for the care of experimental animals. All procedures of the current experiment have been approved by the Committee of the Faculty of Science of Assiut University, Egypt.

3. Results and discussion

3.1. Chemistry of the compounds

5-Methyl-2-phenyl-2,4-dihydropyrazol-3-one **(1)**, which was synthesized according to literature procedure, was subjected to react with Vilsmeier's reagent, afforded 5-chloro-3-methyl-1-phenyl-*1H*-pyrazol-4-carbaldehyde **(2)**.When the pyrazolecarbaldehyde **2** was allowed to react with hydroxyl amine hydrochloride in ethanol in the presence of fused sodium acetate, the corresponding oxime **3** was obtained. The latter compound was dehydrated using acetic anhydride into the corresponding 5-chloro-3-methyl-1-phenyl-*1H*-pyrazole-4-carbonitrile derivative (**4**) [29].

The attempt to synthesize thieno[2,3-c]pyrazole through converting chloropyrazolecarbonitrile**4** into mercaptopyrazole**5** using thiourea in ethanol, as with other moieties, followed by reaction with α-halogenated compounds, failed. This forced us to search about other method for synthesizing thienopyrazole compound **7**. The desired result was achieved by the reaction of elemental sulfur with chloropyrazolecarbonitrile**4** in the presence of sodium borohydride through reduction of sulfur in ethanol to afford not isolated intermediate sodium salt C, which was used *in situ* in the next reaction with α-halogenated compounds to afford *S*-alkylated mercaptopyrazole carbonitrile compounds **6a–f**. Compounds **6a–f** underwent *Thorpe-Ziegler* cyclization upon heating in ethanolic sodium ethoxide solution to afford thienopyrazoles**7 a–f**. Conversion of **6b–7b** was proved by spectral analysis, ^{1}H NMR revealed the disappearance of signal at 3.30 characteristic for -CH_2- in compound **6**, and appearance of signal at 6.90Characteristic for NH_2. Also, the IR of **7b** revealed the disappearance of absorption band characteristic for CN group at 2210 cm^{-1} in compound **6b** and appearance of bands characteristic for NH_2 group. Mass spectrum of compound **6b** showed molecular ion peak at 272.14 and showed the base peak at 255.77 that is mean that the molecular peak ion peak loss one molecule of ammonia to give the base peak (Scheme 1).

On the other hand, the reaction of 4-amino-3-methyl-1-phenyl-*1H*-thieno[2,3-c]-pyrazole-5-carboxamide (**7b**) with chloroacetyl chloride in dioxane followed by neutralization with sodium carbonate solution afforded the chloroacetyl amino compound **8**. The structure of the latter compound was proven by elemental and spectral analysis. IR spectrum revealed the appearance of absorption band at 1690, 1650 cm^{-1} characteristic for two carbonyl groups. ^{1}H NMR spectrum in (DMSO-d_6) showed two singlet signals at δ 4.50, 10.50 ppm characteristic for CH_2 and NH groups respectively.

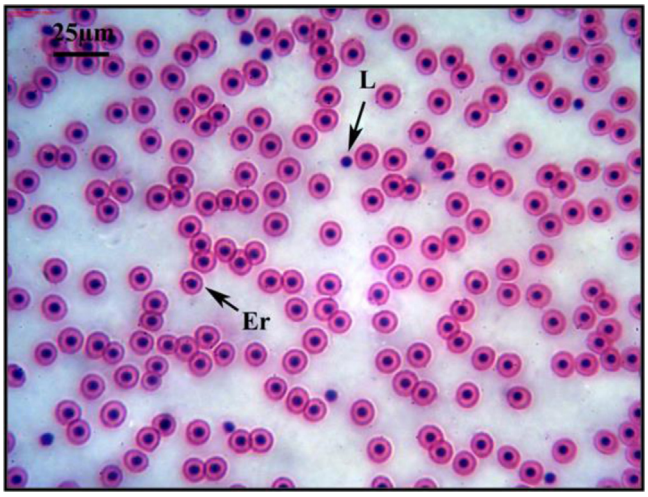
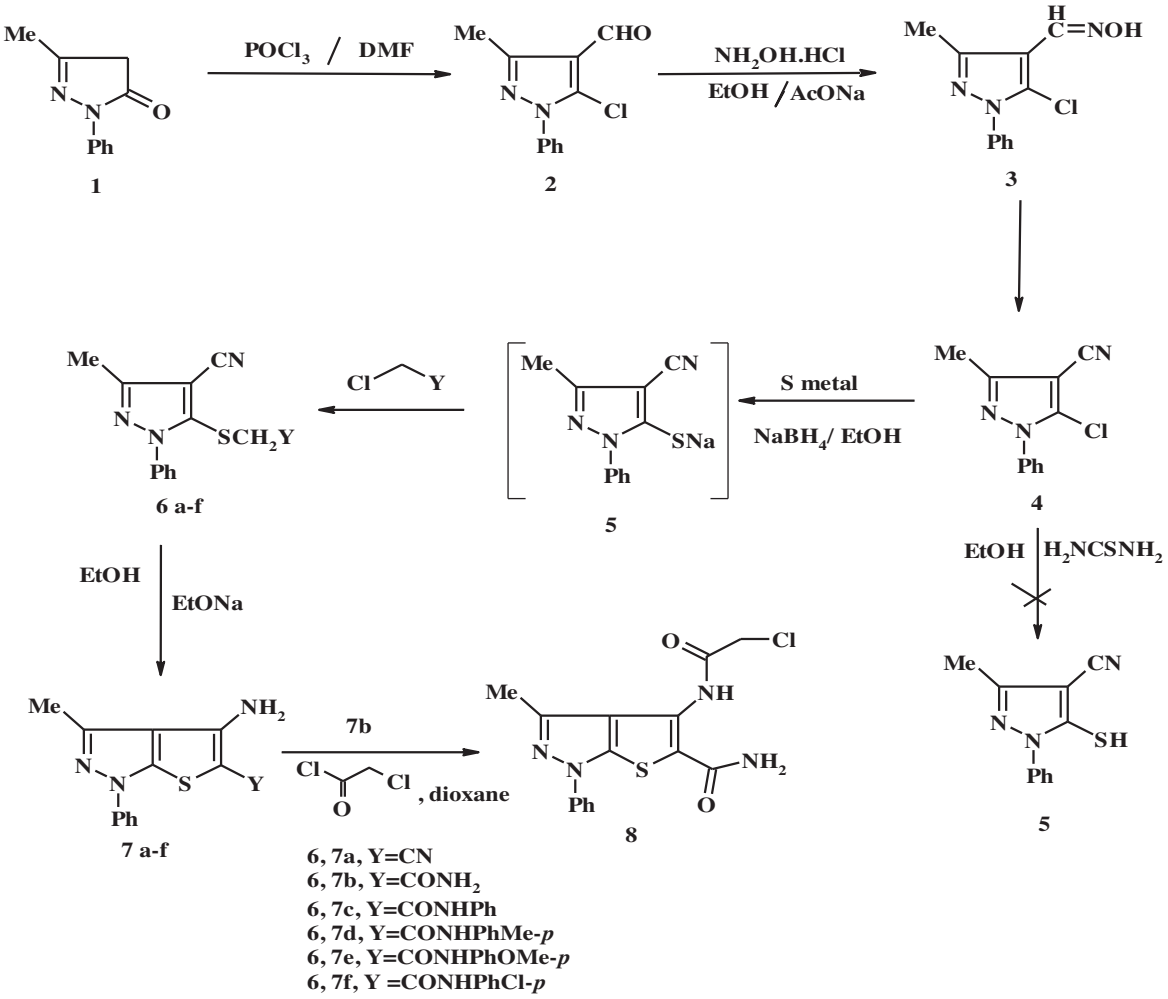


Fig. 1. Blood film of control catfish *Clarias gariepinus* showing the rounded shape of the nucleated erythrocytes (Er) and leucocytes (L). (H&E, ×400).

3.2. Biological activity evaluation

The newly synthesized compounds **6 a–f** and **7a–f** were evaluated for potential biological activities through biomarkers assays (erythrocytes alterations). The results are presented in Table 4 and Figs. 1–7. As shown in Table 4, the altered erythrocyte percentage appears in 4-nonylpenol group is more than the control one. This percentage was slightly decreased in groups of 4-nonylphenol which in combination with the newly synthesized thieno[2,3-c]pyrazoles in comparison with group exposed to 4-nonylphenol only. The present results were similar to that of [32]. The percentage of the altered erythrocytes was 0.6, 1, 3.7, 12, 28, 29, and 40 % for 4-NP in combination with 4-amino-3-methyl-1-phenyl-1*H*-thieno[2,3-c]pyrazole-5-carboxamide **(7b)**, control & 4-NP in combination with 4-amino-*N*-(4-chlorophenyl)-3-methyl-1-phenyl-1*H*-thieno[2,3-c]pyrazole-5-carboxamide **(7f)** & 4-NP in combination with 4-amino-3-methyl-1-phenyl-1*H*-thieno[2,3-c]pyrazole-5-carbonitrile **(7a)** & 4-NP in combination with 4-amino-*N*-(4-methoxyphenyl)-3-methyl-1-phenyl-1*H*-thieno[2,3-c]pyrazole-5-carboxamide **(7e)** & 4-NP in combination with 4-(2-chloroacetamido)-3-methyl-1-phenyl-1*H*-thieno[2,3-c]pyrazole-5-carbox- amide **(8)** and 4-NP only groups respectively. In this aspect, the improvement of the alterations in RBC's of fish was reported after treatment with antioxidants as lycopene [53]. Quince leaf extract [34], and Vit. E [54]. Our results using thieno[2,3-c]pyrazole are used to improve the effects of 4-nonylphenol, which showed more effective role in the decreased number of the altered RBC's. Although, all the synthesized thienopyrazole derivatives were found to be active, the 4-amino carboxamide compound **7b** showed a potent antioxidant potential against 4-nonylphenol toxicity and was found to be more active than the other synthesized



Scheme 1. Synthesis of 4-amino-3-methyl-5-substituted-1-phenyl-1*H*-thieno[2,3-c]pyrazoles (7a–f).

Table 4
Altered erythrocytes in the African Catfish *Clarias gariepinus* exposed to 4-nonylphenol in combination with thieno[2,3-c]pyrazole compounds. The data are presented as means ± S.E. (range). *N* = 9.

Parameters	Control	4-Nonylphenol	4-Nonylphenol with amino-carbonitrile (7a)	4-Nonylphenol with amino carboxamide (7b)	4-Nonylphenol with amino-*N*-(4-methoxyphenyl)carboxamide (7e)	4-Nonylphenol with amino-*N*-(4-chlorophenyl) carboxamide (7f)	4-Nonylphenol with 4-(2-chloroacetamido)-3-methyl-1-phenyl-1*H*-thieno[2,3-c]pyrazole-5-carboxamide (8)
Altered erythrocytes %	1 ± 0.3 (0–3)[d]	40.3 ± 4.87 (12–62)[a]	12 ± 1.03 (5–18)[c]	0.6 ± 0.16 (0–1)[d]	28.3 ± 2.04 (15–37)[b]	3.7 ± 0.37 (2–5)[cd]	29.1 ± 3.05 (14–43)[b]

compounds, which showed several active folds more than the chloroacetamido derivative **8**.

Fig. 1 shows the blood smear of normal fish and represented the normal structure of blood of the African catfish; *C. gariepinus*. The blood was composed of nucleated erythrocytes (Er); rounded with a centrally located rounded nucleus and leucocytes (L), they stained bright purple, their nucleus have no definite shape, being sometimes rounded, kidney-shaped or lobulated. Exposure of fish to sub lethal concentration of 4-nonylphenol resulted in morphological changes in the red blood cells and appearance of some pathologic types of cells that are sickle cells (Sk) which vary in shape between elliposoidal, boat-shaped and genuine sickles, swelled cells and tear drop like cells (Tr), their shape looks like tear with pointed apices (Fig. 2). All of those alterations were recorded in the previous work on the same species under the effects of UVA [39], 4-nonylphenol [32] and 2,4-dichlorophenoxyacetic acid and butachlor [55].

Although observed improvement was recorded in all compounds in combination with 4-nonylphenol; injection of 4-amino thieno[2,3-c]pyrazolecarbonitrile (**7a**) to the fish treated with 4-nonylphenol showed little improvement in the red blood cells where some alterations such as swelled cells, sickle cells (Sk), acanthocyte (Ac), crenated cells; where the red blood cells develop irregular cell surface with numerous projections and cells have prominent vacuoles (**Va**) still appeared in the blood film (Fig. 3). An improvement in the red blood cells morphology was appeared in the group of fish treated with 4-nonylphenol in combination with the amino-carboxamide compound **7b** (Fig. 4) and the group treated with 4-nonylphenol in combi-

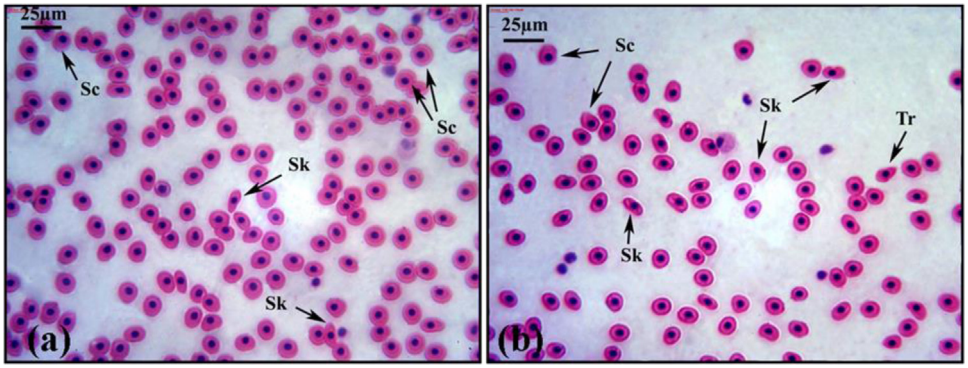


Fig. 2. Blood film of catfish *Clarias gariepinus* treated with 1 mg/l 4-nonylphenol: (a) & (b) showing swelled cells (Sc), sickle cells (Sk) and tear drop like cells (Tr) (H&E, ×400).

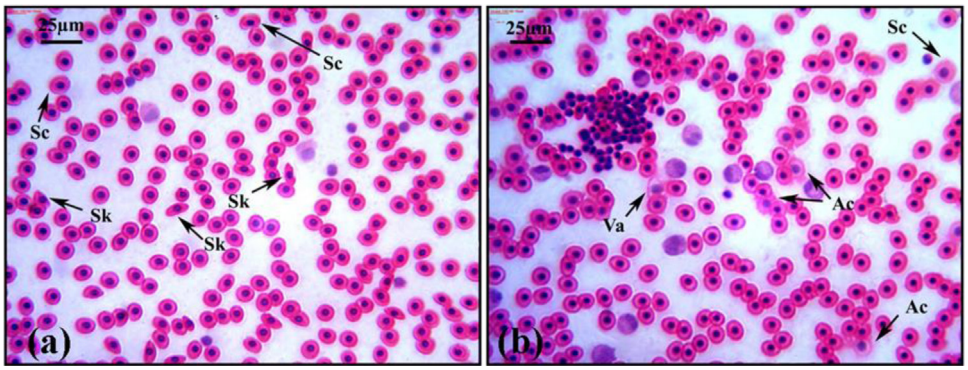


Fig. 3. Blood film of catfish *Clarias gariepinus* treated with 4-nonylphenol plus**4-**amino-3-methyl-1-phenyl-1*H*-thieno[2,3-c]pyrazole-5-carbonitrile: **(7a)** (a) & (b) showing swelled cells (Sc), sickle cells (Sk), acanthocytecrenated cells (Ac) and cells have prominent vacuoles (Va). (H&E, ×400).

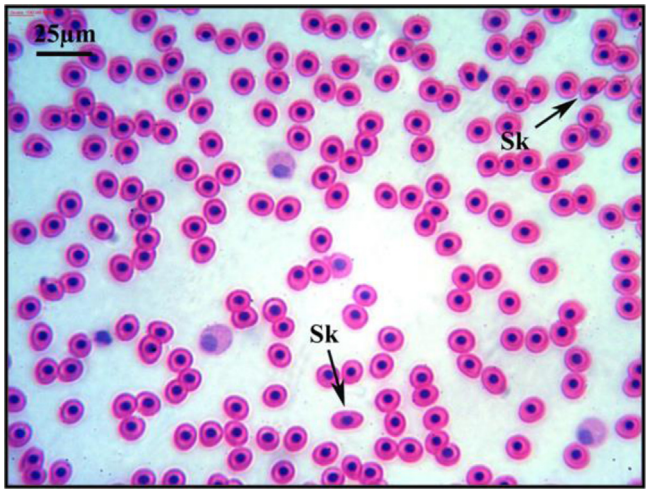


Fig. 4. Blood film of catfish *Clarias gariepinus* treated with 4-nonylphenol plus4-amino-3-methyl-1-phenyl-1*H*-thieno[2,3-c]pyrazole-5-carboxamide **(7b)** showing normal erythrocytes with presence of few numbers of sickle cells (Sk). (H&E, ×400).

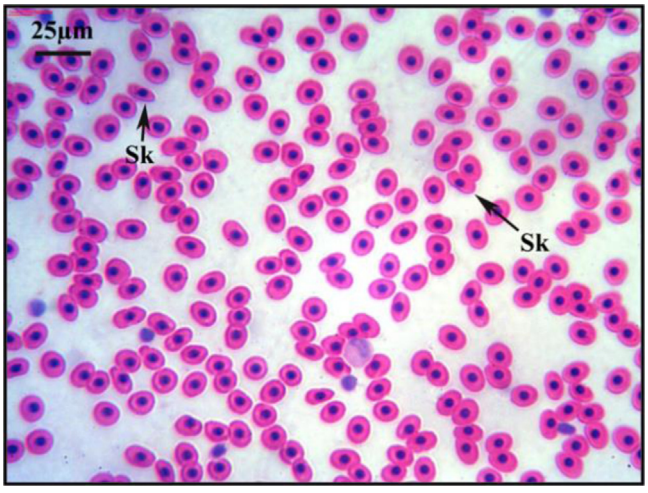


Fig. 5. Blood film of catfish *Clarias gariepinus* treated with 4-nonylphenol plus 4-amino-3-methyl-*N*-phenyl- *p*-chlorophenyl-1*H*-thieno[2,3-c]pyrazole-5-carboxamide **(VIIf)** showing normal erythrocytes with presence of few numbers of sickle cells (Sk). (H&E, ×400).

nation with 4-amino-*N*-(*p*-chlorophenyl)-3-methyl-1-phenyl-1*H*-thieno[2,3-c]pyrazole-5-carboxamide (**7f**) (Fig. 5) compared to the treated fishes with 4-nonylphenol only, in those groups the red blood cells retained toit's the normal shape with presence of few numbers of sickle cells (Sk).

Some of abnormal shapes of the red blood cells were recorded in the group treated with 4-nonylphenol in combination with the amino-*N*-(*p*-methoxyphenyl)carboxamide (**7e**) (Fig. 6) and other group treated with 4-nonylphenol in combination with the chloroacetamido compound **8** (Fig. 7). So that, the antioxidant effects of the latter compounds were less than the other compounds, but more than the treated 4-nonylphenol group only. All the synthesized compounds **6 a–f** and **7 a–f** were found to be non cytotoxic and showed antioxidant role against toxicity of 4-nonylphenol. Pyrazolone derivatives are an important class of heterocyclic compounds that occur in many drugs and synthetic products [56]. Pyrazolone is a biologically important scaffold associated with multiple pharmacological activities such as antitubercular [57,58], antifungal [59,60], antibacterial, anti-inflammatory [61–64], anti-tumor activities [65–67], antidiabetic [68], antiviral [69,70], and antioxidant [71].

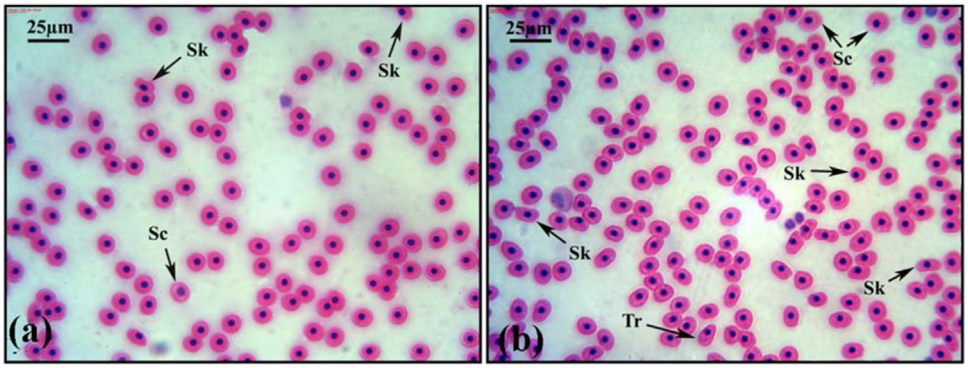


Fig. 6. Blood film of catfish *Clarias gariepinus* treated with 4-nonylphenol plus: 4-amino-3-methyl-1-phenyl-*N*-*p*-anisyl-1*H*-thieno[2,3-c]pyrazole-5-carboxamide **(7e)** (a) & (b) showing swelled cells (Sc), sickle cells (Sk) and tear drop like cells (Tr). (H&E, ×400).

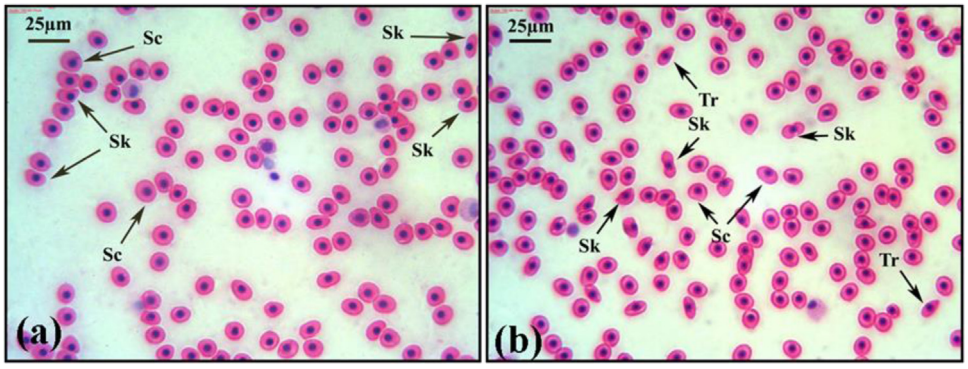


Fig. 7. Blood film of catfish *Clarias gariepinus* treated with 4-nonylphenol plus 4-(2-chloro-acetylamino)-3-methyl-1-phenyl-1*H*-thieno[2,3-c]pyrazole-5-carboxamide **(8)**: (a) & (b) showing swelled cells (Sc), sickle cells (Sk) and tear drop like cells (Tr). (H&E, ×400).

4. Conclusions

In conclusion, we have successfully developed an easy practical access to novel and readily accessible method for the synthesis of biologically important thieno[2,3-c]pyrazole compounds. These compounds were evaluated for their biological activities in various biological assays. The potent role of compounds indicates their potential as possible leads for the treatment of oxidative stress and repair the damage resulted due to toxicity of chemical pollutants (4-nonylphenol). The most promising results of those compounds can serve as templates for the new studies in vitro and in vivo.

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