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SYNTHESIS OF SOME NOVEL PYRAZOLINE AND PYRAZOLE DERIVATIVES AS POTENTIAL *IN VITRO* ANTI-XANTHINE OXIDASE AND ANTIOXIDANT ACTIVITIES

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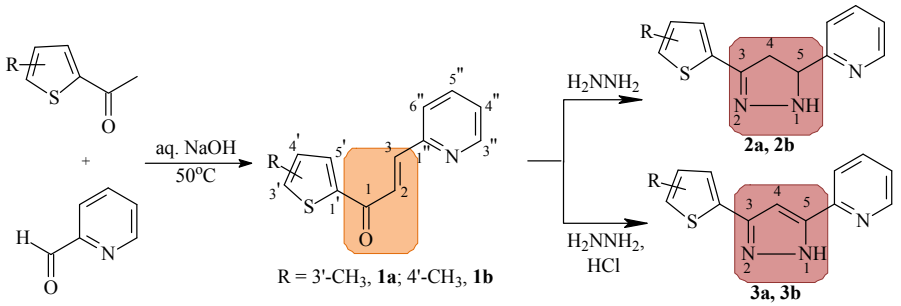
2-Pyrazolines and 2-pyrazole, electron-rich nitrogen containing heterocyclic systems, play an important role in several biological and pharmacological activities. In this paper, we report the synthesis of novel 3,5-disubtituted-2-pyrazoline and pyrazole derivatives (**2a-b**, **3a-b**) starting from azachalcones (**1a, 1b**). The structure of the synthesised compounds were judged by ^{1}H NMR, ^{13}C NMR and IR. The synthesised 3,5-disubtituted-2-pyrazoline and pyrazole derivatives were evaluated *in vitro* for their xanthine oxidase (XO) inhibitory activities, with most of the investigated compounds being shown to be potent inhibitors of bovine milk XO. Antioxidant activities of the synthesised compounds (**1a, 1b, 2a-b**, **3a-b**) were determined with CUPric Reducing Antioxidant Capacity (CUPRAC), ABTS (2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)/Persulphate and DPPH (1,1-diphenyl-2-picrylhydrazyl) assays. Pyrazoline and pyrazol derivatives also revealed notable antioxidant activities in DPPH scavenging (SC_{50}: 9.91-15.16 µg/mL) and cupric reducing/antioxidant capacity (5.68-10.56 mM TEAC) tests. Also, compounds *3-(3-methylthiophen-2-yl)-5-(2-pyridinyl)-1H-pyrazoline* (**2a**) and *3-(4-methylthiophen-2-yl)-5-(2-pyridinyl)-1H-pyrazoline* (**2b**) were found to be more potent radical scavenging activity than butylated hydroxy toluene (BHT) to $ABTS^{\bullet+}$ radical cation decolorisation assay.



INTRODUCTION

1,3-diphenylprop-2-en-1-one is well known by the generic term “chalcone”. These compounds, members of the flavonoids family, are an important group of natural and synthetic products. Synthetic chalcones are commonly synthesised via Claisen-Schmidt condensation between a ketone and an aldehyde catalysed by bases and acids.[1-7] Both chalcones and flavones have recently become the focus of attention due to their wide range of biological and pharmacological properties.[1-3] Azachalcones are chalcone analogues which contain an annular N-atom in the phenyl ring, giving rise to a pyridyl moiety. These compounds have a lot of biological effects such as anti-tuberculosis, anti-microbial, anti-oxidant, anti-HIV, anti-fungal, anti-inflammatory, and anti-bacterial properties.[1-8]

2-Pyrazolines and 2-pyrazoles are the important members of heterocyclic compounds comprising two nitrogen atoms in vicinal positions. In the literature, there are different methods for the synthesis of these compounds but the most

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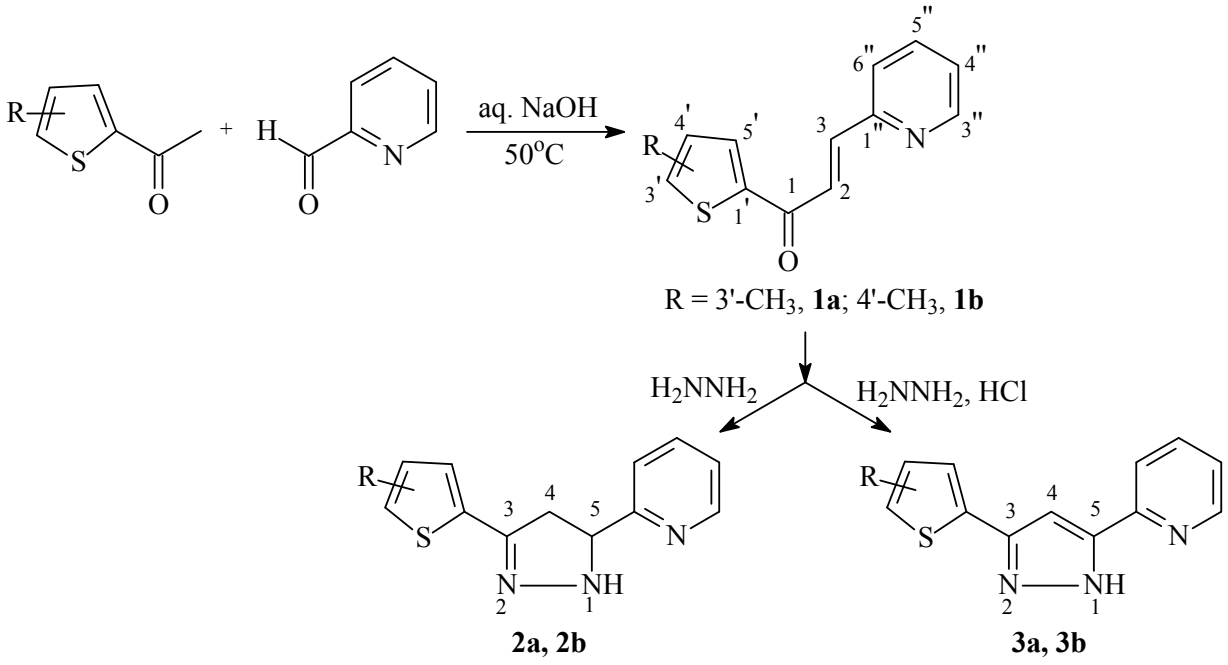
common and easiest synthesis route of target molecules is cycloaddition reactions of α,β-unsaturated aldehydes and ketones with hydrazine hydrate and their derivatives.[9-12] Chalcones are well known intermediates for the synthesis of various heterocyclic compounds, especially five and six-membered heterocyclic compounds[13-15] and widely preferred as starting material due to the versatility of their 1,3-diaryl-2-propen-1-one carbon skeleton for the synthesis of 2-pyrazolines and 2-pyrazole.[15-17] Target compounds have biological activities such as anti-microbial, analgesic, anti-cancer, anti-depressant, anti-convulsant, anti-inflammatory, anti-tubercular, anti-hyperglycaemic, anti-oxidant and herbicidal properties, among others.[15-22] The pyrazoline and pyrazole rings are present as the core in a variety of leading drugs such as Celebrex, Rimonabant etc.[21]

Free radicals are mainly generated by metabolic processes in the human body, and some radicals have essential roles in normal cell processes such as neural signal transduction.[22] On the other hand, excessive free radical incursion, causing very important diseases, can damage all components of the cell including DNA, proteins and lipids.[23] Antioxidants are chemicals that can react with free radicals and terminate their chain reactions, by which damage to essential biomolecules is prevented. As oxidative stress plays an important role in many diseases, such as cancer, Parkinson's, Alzheimer disease, heart failure and stroke, the use of antioxidants is intensively studied in medicinal chemistry, particularly as a means for the treatment of these widespread diseases.[24,25]

Xanthine oxidase (XO) is a key enzyme that belongs to the molybdenum-protein family. Molybdenum catalyses the oxidation of xanthine and hypoxanthine into uric acid.[26] Elevated concentrations of uric acid in the bloodstream in humans leads to the formation of gout, characterised by hyperuricaemia and recurrent attacks of arthritis.[27,28] Allopurinol is a potent inhibitor of xanthine oxidase used to treat gout and hyperuricaemia.[29] However, severe toxicity of this inhibitor such as vasculitis, rashes, eosinophilia, hepatitis has been reported,[30] hypersensitivity problems, Steven-Johnson syndrome, renal toxicity and fatal liver necrosis.[31-34] Therefore, it is necessary to develop a new inhibitor without undesirable side effects.

A literature survey has revealed that when a biodynamic heterocyclic system was combined with another, the obtained molecule had enhanced activity.[15] Also, *in vitro* screening of heteroaryls such as furan, thiophene or pyridyl substituted for the pyrazole and pyrazoline is critical for the higher antioxidant and XO inhibitory activity.[35] Therefore, as part of our continuous studies in this area, a series of four new 3,5-disubtituted-2-pyrazoline and 2-pyrazole derivatives (**2a-b, 3a-b)** starting from azachalcones (**1a, 1b**) was used. Then, all new compounds were investigated for their anti-XO inhibitory properties and antioxidant activities.



Scheme 1 – The synthetic pathway for target compounds.

RESULTS AND DISCUSSION

The aim of this study is to synthesise 3,5-disubstituted-2-pyrazoline and 2-pyrazole (**2a-b, 3a-b**) from methyl substituted azachalcone (**1a, 1b**), which is a natural chalcone analogue. The synthetic routes of all of the targeted compounds are shown in Scheme 1. The structures of all of the newly synthesised compounds were fully determined by ^{1}H NMR, APT, IR spectra.

The starting compounds (2*E*)-1-(3-/4-methylthiophen-2-yl)-3-(pyridin-2-yl)prop-2-en-1-one (**1a, 1b**) were prepared by Claisen-Schmidt condensation in the presence of basic catalysts such as NaOH according to the procedures reported in the literature.[6,7] The IR spectra of **1a, 1b** exhibited a band due to the unsaturated carbonyl group at 1640-1646 cm^{-1}. The most noticeable feature of the structural characterisation of compounds **1a, 1b** was the assignment of the ^{1}H-NMR resonances of the α,β-unsaturated C=C bond. Based on $^3J_{(\alpha,\beta)}$ values of 14.0 and 16.0 Hz, respectively, the (*E*)-configuration was assigned to **1a-1b**.[1-7] The ^{13}C NMR of the synthesised azachalcones showed signals at around 180 ppm corresponding to the C=O group.

In the last step, the condensation of chalcones with hydrazine monohydrate (95%) (acid-free and in the presence of concentrated HCl) at boiling in absolute ethanol were completed within 12 h to obtain the target compounds. At the end of the reactions, two new pyrazolines [3-(3-/4-methyl-thiophen-2-yl)-5-(2-pyridinyl)-1H-pyrazoline] (**2a, 2b**) and two new pyrazoles [3-(3-/4-methylthio-phen-2-yl)-5-(2-pyridinyl)pyrazole] (**3a, 3b)** were synthesised.

In the ^{1}H NMR spectra of compounds **2a, 2b**, the peaks were split to a doublet of doublets and appear in the rangej 3.18–3.54 ppm; the merged doublet of doublets at around 4.58 ppm indicated the newly composed geminal –CH_2– protons and –CH– proton owing to the pyrazoline core. This is a characteristic feature of diastereotopic methylene protons and methine protons. This forms an ABX system due to geminal-vicinal coupling between two non-equivalent protons of the methylene group H_a and H_b at C-4 and a methine proton H_x at C-5. Moreover, the broad –NH– peaks appearing at 6.0–7.6 ppm also supported the pyrazoline structures. Values of 39.9, 64.7, 32.3, and 52.0 ppm in APT ^{13}C NMR spectra of compounds (**2a** and **2b,** respectively) confirmed the formation of the pyrazoline fragment. The protons linked to the olefinic carbon atom (C-4) of the pyrazole rings in compounds **3a**, **3b** were observed at 6.87 and 6.85 ppm in their ^{1}H NMR spectra. The chemical shifts in the APT ^{13}C NMR spectra of this carbon atom were shown at 99.9 ppm and 100.2 ppm, respectively. The other protons belonging to the aromatic ring and the aliphatic groups are given with the expected chemical shift and integral values in the experimental section.

CUPRAC antioxidant activity assay

The CUPRAC method of antioxidant capacity measurement was based on the absorbance measurement of the CUPRAC chromophore, Cu(I)-neocuproine (Nc) chelate, formed as a result of the redox reaction of antioxidants with the CUPRAC reagent, bis(neocuproine)copper(II) cation [Cu(II)-Nc], where the absorbance was recorded at the maximal light absorption wavelength of 450 nm. The orange–yellow colour was due to the Cu(I)-Nc charge-transfer complex formed.[44] The best antioxidant capacity in the CUPRAC method was observed for compound **2b** with 10.56±0.19 mM TEAC/mg compound (Table 1). Also, the TEAC value of compound **2a** was calculated as 5.68 ± 0.09 mM TEAC/mg compound (Table 1).

Table 1

TEAC values of the synthesised compounds to CUPRAC antioxidant activity method, nd: not determined

Compound no.	CUPRAC method
	mM TEAC/mg compound
1a	nd
2a	5.68±0.09
3a	nd
1b	nd
2b	10.56±0.19
3b	nd

Similarly to our results, it was reported that Cupric values of the triheterocyclic compounds containing thiophene and 1,2,4-triazole groups ranged from 0.400±0.072 to 1.476±0.025 mg TEAC/mg compounds. The most active compound from this study had a TEAC value with 1.476± 0.025 mg TEAC/mg compounds according to the CUPAC method.[39] In another study, it was stated that triazole derivatives containing the thiophene group were highly active in the FRAP assay, 503-1257 mM TEAC values.[45] Also, it was reported that TEAC values of benzimidazole derivatives com-

pounds containing a triazole nucleus ranged from 4.16 to 8.67 mM TEAC/mg compound.[39]

DPPH scavenging assay

DPPH is often used as a reagent to evaluate the free radical scavenging activity of antioxidants. In the DPPH assay, the antioxidants were able to reduce the stable radical DPPH to the yellow coloured diphenylpicrylhydrazine.[46,47] The colour changed from purple to yellow after reduction, which can be quantified by its decrease in absorbance at a wavelength of 517 nm. The results were expressed as SC_{50} (μg/mL, Figure 1). When the CUPRAC and DPPH scavenging activity methods were correlated by each other, compound **2b** could be seen as an efficient sample with an SC_{50} value of 9.91 μg/mL for the DPPH method. Compound **2a** had good DPPH radical scavenging activity.

It was determined that the SC_{50} values of compounds containing a 1,2,4-triazole ring ranged from 3.91 to 16.75 μg/mL for the DPPH method.[39] One of these compounds had the lowest SC_{50} value of 3.91μg/mL, because of the highest antioxidant capacity and radical scavenging activity.[39] In another study, the compounds containing oxadiazole and thiosemicarbazide were also effective DPPH radical scavengers, with SC_{50} values of 19.34, 77.36, 13.46 and 13.27 μg/mL respectively.[42] It was shown that triazole derivatives were highly active in the DPPH method with 69.0–88.2% DPPH radical scavenging activity.[45] It was reported that the SC_{50} values of benzimidazole derivatives compounds containing a triazole nucleus were highly effective for the DPPH method, with SC_{50} values ranging from 7.03 to 31.27 μg/mL.[49]

$ABTS^{\bullet+}$ radical scavenging activity

The pre-formed radical monocation of 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) was generated by the oxidation of ABTS with potassium persulphate and is reduced in the presence of such hydrogen-donating antioxidants. All of the tested compounds exhibited effective radical cation scavenging activity. As seen in Table 2, **2a** and **2b** are effective $ABTS^{\bullet+}$ radical scavengers in a concentration-dependent manner (5.0–0.31 μg/mL). There is a significant decrease in the concentration of $ABTS^{\bullet+}$ due to the scavenging capacity for all concentrations of compound **2b**. Compound **2b** showed more efficient scavenging activity than BHT and ascorbic acid, at all concentrations. The scavenging effect of compound **2b** and standards on $ABTS^{\bullet+}$ decreased in the following order: **2b**≈Catechin>Ascorbic Acid≈**2a** >BHT (91.29, 91.07, 90.91, 90.71and 88.34%, respectively) at the same concentration (5.0 μg/mL). Also, compound **2a** exhibited very good scavenging activity at the same concentration (Table 2).

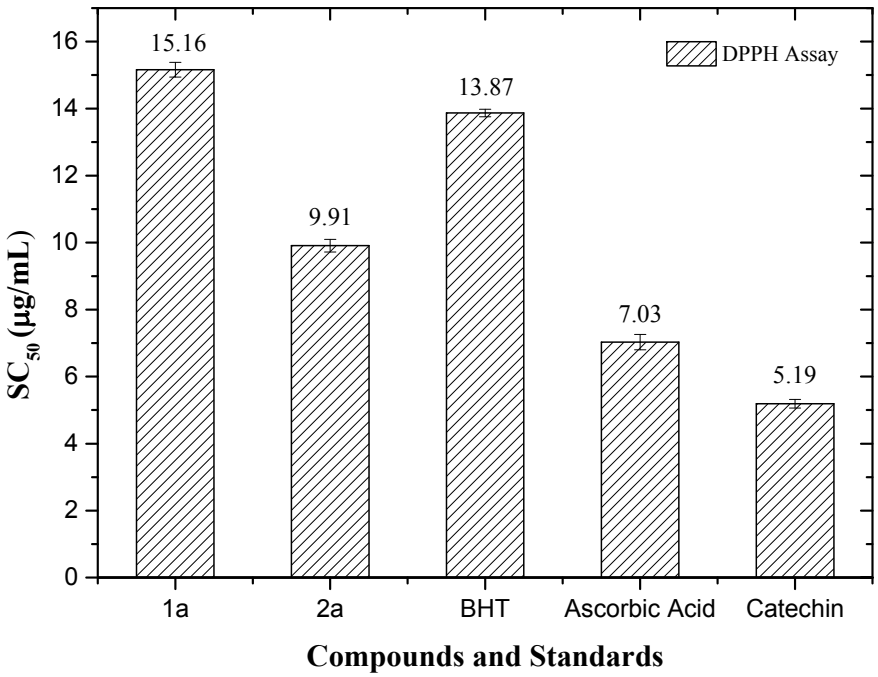


Fig. 1 – SC_{50} values (μg/mL) of the synthesised compounds and standards to DPPH method.

Table 2

ABTS$^{\bullet+}$ Radical scavenging activity values (%)
of the synthesised compounds and standards at various final concentrations

Compound	ABTS Radical Scavenging (%)				
	5.00 (μg/mL)	2.50 (μg/mL)	1.25 (μg/mL)	0.63 (μg/mL)	0.31 (μg/mL)
2a	90.71%	69.86%	34.29%	23.00%	14.14%
2b	91.29%	82.00%	64.00%	35.71%	18.43%
Catechin	91.07%	89.29%	78.66%	44.34%	21.29%
Ascorbic Acid	90.91%	61.82%	39.55%	23.21%	16.62%
BHT	88.34%	56.21%	25.91%	18.39%	9.98%

Similar to our ABTS$^{\bullet+}$ radical scavenging results, a number of benzimidazole derivative compounds, containing different groups such as thiophene, 1,2,4-triazole rings,[39] salicyl, oxadiazole, thiosemicarbazide[42] and 1,2,4-triazole and fluoro, have been reported to show good ABTS$^{\bullet+}$ radical scavenging activity. In these studies, it was determined that ABTS$^{\bullet+}$ radical scavenging activity of the benzimidazole derivatives ranged from 91.51% to 53.90%, at a final concentration of 3.13 μg/mL.[39] In another study, it was shown that some compounds containing a 1,2,4-triazole ring exhibited good radical scavenging activity, ranging from 30.70% to 97.80%, at a final concentration of 10.0 μg/mL. SC_{50} values of the benzimidazole derivatives containing salicyl, oxadiazole, thiosemicarbazide and 1,2,4-triazol moieties were between 9.51 and 74.45 μg/mL.[42] In another study, it was calculated that the ABTS$^{\bullet+}$ radical scavenging activity of the benzimidazole derivatives containing a triazole nucleus ranged from 83.80% to 52.46%, at a final concentration of 3.0 μg/mL.[48]

In vitro anti-xanthine oxidase activity

All of the synthesised compounds were evaluated with regard for bovine milk xanthine oxidase inhibitory activity. The results showed that compound **3b** had good activity, capable of inhibiting XO by up to 100.00% at a concentration of 8.0 μg/mL (Table 3, Figure 2). Among the synthesised compounds, **3b** displayed the best inhibitory effect against XO with an IC_{50} value of 1.03±0.23 μg/mL. In addition to these good results, the IC_{50} value of allopurinol was determined to be 0.55±0.09 μg/mL. Compounds **1a** and **3a** exhibited a very potent inhibitory effect against XO. Compound **2a** showed good XO enzyme inhibition activity.

In the literature, it was reported that caffeic acid and chlorogenic acid had IC_{50} values of about 74.6 ± 11.04 mM and 126.28 ± 2.86 mM, respectively.[49] The IC_{50} values of allopurinol, luteolin, apigenin, kaempferol and quercetin were 3.65, 1.49, 2.37, 3.35, and 2.34 mM, respectively.[50] It was determined that Allopurinol had an IC_{50} value of about 3.74 mg/mL.[51] Another published study reported that 4-(4-Bromophenyl)-5-{[5,6-dichloro-2-(3,4-dichlorobenzyl)-1H-benzimidazol-1-yl]methyl}-4H-1,2,4-triazole-3-thiol showed promising activity to inhibit XO by up to 99.56% at a concentration of 125 μg/mL, with an IC_{50} value of 33.87±0.46 μM.[52] It was reported that compound **4a** fluorine containing a 1,2,4-triazole-5-one derivative, had good activity to inhibit XO by up to 92.13% at a concentration of 31.25 μg/mL, with an IC_{50} value of 36.37±0.11 μM.[53]

Table 3

The results of anti-XO activity and IC_{50} values (μg/mL)
of the synthesised compounds and Allopurinol as standard inhibitor

% Inhibition XO activity		
Compound	**(8.0 μg/mL)**	**IC_{50} (μg/mL)**
1a	99.81±0.99	1.61±0.29
2a	89.38±1.17	4.15±0.11
3a	100.00±0.04	1.43±0.48

Table 3 (continued)

1b	98.88±0.81	2.88±0.07
2b	19.21±0.57	nd
3b	100.00±0.06	1.03±0.23
Allopurinol	100.00±0.00	0.55±0.09

nd; not determined, Control, bovine milk xanthine oxidase without inhibitor; Allopurinol, positive control.

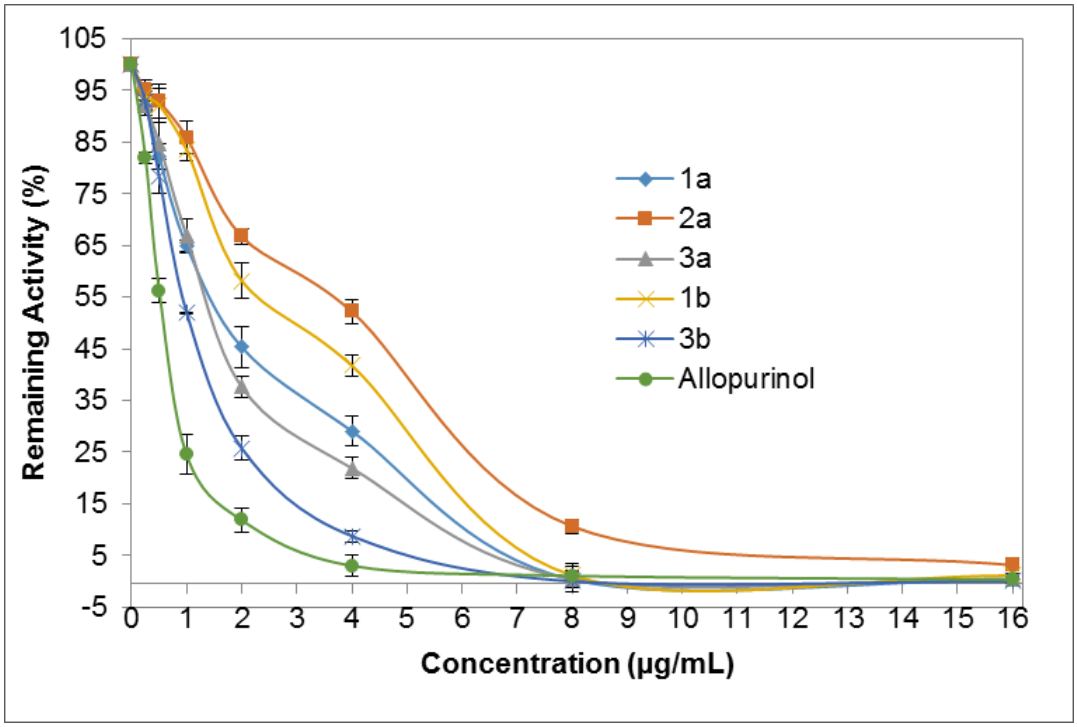


Fig. 2 – Dose-dependent inhibitory effect of compounds and allopurinol. Allopurinol was used as a standard inhibitor. All compounds and allopurinol were measured at the final concentration of 8.0 µg/mL. Residual activities of compounds are expressed as the mean ±SD in triplicate.

EXPERIMENTAL

All of the chemicals were supplied by Merck (Darmstadt, Germany) and used without further purification. The solvents ($CHCl_3$, *n*-hexane, EtOH, MeOH, AcOEt, and Et_2O) used were either of analytical grade or bulk solvents distilled before use. NMR spectra were recorded on a Varian-Mercury 400 MHz spectrophotometer (Varian, Darmstadt, Germany) in $CDCl_3$-d_1 (99.8% at. D), using TMS as an internal control. The NMR data assignment was based on ^1H, ^{13}C, APT, and ACD NMR software (Toronto, Canada). IR spectra were obtained with a Perkin-Elmer 100 FT-IR (4000–400 cm^{-1}) spectrophotometer (Waltham, MA, USA). Melting points were determined using a Thermo-var apparatus (Braunschweig, Germany) fitted with a microscope and are shown uncorrected. TLC was carried out on Merck (Darmstadt, Germany) pre-coated 60 Kieselgel F_{254} analytical aluminium plates. The purity of all of the synthesised compounds was checked by TLC after purification by column chromatography and crystallisation; spots were detected by exposure to a UV lamp at 254 nm for a few seconds. Silica gel 60 (0.040-0.063 mm) was used in column chromatography.

General procedure for the synthesis of compounds 1a and 1b

The heterocyclic azachalcones (**1a**, **1b**) were readily prepared by condensation of the 2-pyridine carbaldehyde with 2-acetyl-5-methylthiophene or 2-acetyl-4-methylthiophene. Briefly, to a solution of NaOH (0.02 mol) in 50 mL dist. water, aromatic ketone (0.01 mol) was added. Then, an appropriate amount of pyridine carbaldehyde (0.01 mol for each) solution in EtOH (3 mL) was added drop by drop. After the addition was completed, the reaction mixture was stirred at 50°C (TLC control). The aqueous phase was extracted with $CHCl_3$ (3 x 30 mL). The combined organic phases were dried over Na_2SO_4 and the solvent was removed using a rotary evaporator. The residue was purified by column chromatography on a silica gel (4:5, n-hexane:EtOAc).

(2E)-1-(3-methylthiophen-2-yl)-3-(pyridin-2-yl)prop-2-en-1-one (**1a**):

Yield 88%, mp: 85-87°C, FT-IR (ATR) (ν_{max}, cm^{-1}): 3077, 2918, 1640, 1587, 1444, 1434, 1335, 1242, 985, 776, ^1H NMR ($CDCl_3$, 400 MHz) δ (ppm): 2.55 (s,-CH_3), 6.84 (m, 1H, H-4'), 7.27 (m, 1H, H-4"), 7.44 (d, *J*=8.0 Hz, 1H, H-5'), 7.69 (m, 1H, H-6"), 7.71 (m, 1H, H-5"), 7.75 (AB, *J*=14.0 Hz, 1H, H-2),

7.93 (AB, *J*=14.0 Hz, H-3), 8.66 (d, *J*=4.0 Hz, 1H, H-3"). ^{13}C NMR ($CDCl_3$, 100 MHz) δ (ppm): 16.1 (-CH_3), 124.3 (C-4"), 125.1 (C-6"), 125.4 (C-4'), 127.0 (C-2), 133.2 (C-5'), 136.8 (C-5"), 141.4 (C-3), 143.0 (C-3'), 150.1 (C-3"), 151.1 (C-1'), 153.0 (C-1"), 181.5 (C-1).

(2E)-1-(3-methylthiophen-2-yl)-3-(pyridin-2-yl)prop-2-en-1-one (**1b**):

Yield 82%, mp: 70-72°C, FT-IR (ATR) (ν_{max}, cm^{-1}): 3047, 2926, 1646, 1596, 1579, 1429, 1416, 1325, 1202, 998, 772, ^{1}H NMR ($CDCl_3$,400 MHz) δ (ppm): 2.55 (s,-CH_3), 7.25 (bs, 1H, H-3'), 7.27 (m, 1H, H-4"), 7.43 (bs, 1H, H-5'), 7.71 (m, 1H, H-6"), 7.73 (m, 1H, H-5"), 7.76 (AB, *J*=16.0 Hz, 1H, H-2), 7.94 (AB, *J*=16.0 Hz, H-3), 8.66 (d, *J*=4.0 Hz, 1H, H-3"). ^{13}C NMR ($CDCl_3$, 100 MHz) δ (ppm): 15.6 (-CH_3), 124.4 (C-3'), 125.3 (C-4"), 125.5 (C-6"), 130.3 (C-2), 134.5 (C-5'), 136.9 (C-5"), 139.0 (C-4'), 141.8 (C-3), 145.9 (C-1'), 150.1 (C-3"), 153.0 (C-1"), 182.0 (C-1).

General procedure for the synthesis of compounds 2a and 2b

To a solution of the appropriate azachalcone, **1a** or **1b** (5.0 mmol) in absolute ethanol (15 mL) and hydrazine monohydrate (5.0 mmol) was added. The reaction mixture was heated under reflux for 6-12 h; then, on completion of the reaction, followed by TLC examination, this was allowed to cool to room temperature. The separated crystalline solid was filtered, washed with ice water, and dried. As a result, solid products were obtained which were recrystallised using ethanol.

3-(3-methylthiophen-2-yl)-5-(2-pyridinyl)-1H-pyrazoline (**2a**):

Yield 80%, mp: 110-112°C, FT-IR (ATR) (ν_{max}, cm^{-1}): 3241, 3068, 2961, 1588, 1566, 1474, 1431, 1330, 1033, 797, ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 2.48 (s,-CH_3), 3.15-3.21 (dd, *J*=16/8 Hz, 1H, H-4_a), 3.50-3.57 (dd, *J*=16/8 Hz, H-4_b), 5.04 (dd, *J*=10/8 Hz, 1H, H-5), 6.66 (d, *J*=3.5 Hz, 1H, H-4'), 6.88 (d, *J*=3.5 Hz, 1H, H-5'), 7.20 (m, 1H, H-4"), 7.44 (d, *J*=7.8 Hz, 1H, H-5"), 7.69 (dt, *J*=7.4/1.5 Hz, 1H, H-6"), 8.56 (d, *J*=4.7 Hz, 1H, H-3"). ^{13}C NMR ($CDCl_3$, 100 MHz) δ (ppm): 15.6 (-CH_3), 39.9 (C-4), 64.7 (C-5), 120.5 (C-6"), 122.6 (C-4"), 125.4 (C-4'), 126.7 (C-5'), 133.8 (C-1'), 137.1 (C-5"), 141.1 (C-3'), 147.5 (C-3), 149.2 (C-3"), 160.0 (C-1").

3-(4-methylthiophen-2-yl)-5-(2-pyridinyl)-1H-pyrazoline (**2b**):

Yield 72%, mp: 98-100°C, FT-IR (ATR) (ν_{max}, cm^{-1}): 3200, 3083, 2922, 1589, 1434, 1372, 1209, 1050, 747, ^{1}H NMR ($CDCl_3$, 400MHz) δ (ppm): 2.20 (s,-CH_3), 3.05-3.00 (dd, *J*=16/8 Hz, 1H, H-4_a), 3.15-3.10 (dd, *J*=16/8 Hz, H-4_b), 4.11 (dd, *J*=10/8 Hz, 1H, H-5), 6.91 (bs, 1H, H-5'), 6.98 (bs, 1H, H-3'), 7.20 (m, 1H, H-4"), 7.23 (m, 1H, H-5"), 7.64 (dt, *J*=7.7/1.9 Hz, 1H, H-6"), 8.56 (d, *J*=8.0 Hz, 1H, H-3"), ^{13}C NMR ($CDCl_3$, 100 MHz) δ (ppm): 15.7 (-CH_3), 32.3 (C-4), 52.0 (C-5), 121.1 (C-3'), 122.1 (C-6"), 125.1 (C-4"), 130.3 (C-5"), 136.7 (C-5'), 137.9 (C-4'), 142.2 (C-1'), 149.3 (C-3"), 154.9 (C-3), 161.5 (C- 1").

General procedure for the synthesis of compounds 3a and 3b

To a solution of the appropriate azachalcone, **1a** or **1b** (5.0 mmol) and hydrazine monohydrate (5.0 mmol) in absolute ethanol (15 mL) containing 0.5 mL of HCl was refluxed for 6-12 h; then, on completion of the reaction, followed by TLC examination, this was allowed to cool to room temperature. The product was purified by column chromatography on silica gel (3:7, Et_2O:EtOAc).

3-(3-methylthiophen-2-yl)-5-(2-pyridinyl)pyrazole (**3a**):

Yield 85%, mp: 79-81°C, FT-IR (ATR) (ν_{max}, cm^{-1}): 3209, 3062, 2915, 2854, 1651, 1592, 1570, 1515, 1472, 1447, 1304, 1218, 1164, 1055, 909, 805, 776, ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 2.48 (s,-CH_3), 6.73 (d, *J*=4.0 Hz, 1H, H-4'), 6.87 (s, 1H, H-4), 7.19 (d, *J*=4.0 Hz, 1H, H-5'), 7.24 (m, 1H, H-4"), 7.69 (d, *J*=8.0 Hz, 1H, H-5"), 7.76 (dt, *J*=8.0/1.7 Hz, 1H, H-6"), 8.65 (d, *J*=4.0 Hz, 1H, H-3"). ^{13}C NMR ($CDCl_3$, 100 MHz) δ (ppm): 15.4 (-CH_3), 99.9 (C-4), 120.1 (C-6"), 123.0 (C-4"), 123.8 (C-4'), 125.7 (C-5"), 133.5 (C-3'), 137.0 (C-5), 137.0 (C-5'), 139.4 (C-3), 139.5 (C-1'), 148.1 (C-1"), 149.5 (C-3").

3-(4-methylthiophen-2-yl)-5-(2-pyridinyl)pyrazole (**3b**):

Yield 82%, mp: 159-161°C; FT-IR (ATR) (ν_{max}, cm^{-1}): 3129, 3062, 2897, 2855, 1597, 1567, 1498, 1448, 1414, 1294, 1175, 1029, ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 2.30 (s,-CH_3), 6.85 (s, 1H, H-4), 6.90 (brs, 1H, H-5'), 7.24 (brs, 1H, H-4'), 7.26 (m, 1H, H-4"), 7.69 (brs, 1H, H-5"), 7.77 (m, 1H, H-6"), 8.67 (d, *J*=4.0 Hz, 1H, H-3"). ^{13}C NMR ($CDCl_3$, 100 MHz) δ (ppm): 15.8 (-CH_3), 100.2 (C-4), 120.0 (C-3'), 120.2 (C-6"), 123.1 (C-4"), 126.3 (C-5"), 135.8 (C-4'), 137.1 (C-5'), 138.1 (C-5), 138.1 (C-3), 138.1 (C-1'), 148.1 (C-1"), 149.5 (C-3").

Biological assays

Antioxidant Activity

In the present study, we demonstrated the antioxidant and radical scavenging mechanism of the synthesised compounds by using different *in vitro* bioanalytical methodologies such as CUPric reducing antioxidant capacity assay, ABTS (2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) and DPPH (1,1- diphenyl-2-picrylhydrazyl) radical scavenging systems. As has been reported in many studies, the activities of natural antioxidants in influencing diseases are closely related to their ability to reduce DNA damage, mutagenesis, carcinogenesis and the inhibition of pathogenic bacterial growth.[36] Antioxidant activity can be considered an index of pharmacological usefulness. It is therefore not a complete surprise that many registered drugs have an antioxidant action which may contribute to their pharmacological activity.[37] Catechin, ascorbic acid and butylated hydroxy toluene (BHT) were used as reference antioxidants.

Cupric reducing antioxidant capacity (CUPRAC) assay

In order to determine the cupric ion (Cu^{2+}) reducing ability of the synthesised compounds, methods previously published in the literature were used.[37,39] Trolox® (Sigma Chemical Co, USA) was also as a standard antioxidant compound and tested under the same conditions. The standard curve was linear between 32 mM and 0. 5 mM Trolox® (r^2=0.9987). CUPRAC values were expressed as mM Trolox® equivalent of 1 mg synthesised compound.

DPPH-free radical scavenging assay

The 1,1-diphenyl-2-picrylhydrazyl DPPH radical scavenging method that is used widely in the model system to investigate the scavenging activities of several synthesised and natural compounds was measured using the method of Brand-Williams.[40] Radical scavenging activity was measured by using ascorbic acid (AA), BHT and catechin (Sigma Chemical

Co, USA) as standards; all values are expressed as SC_{50} (μg compound per mL), the concentration of the samples that causes 50% scavenging of DPPH radicals. The DPPH radical stock solution was prepared fresh daily. The lower SC_{50} values indicate the higher radical scavenging potential.

$ABTS^{\bullet+}$ radical cation decolorisation assay

The ability of the synthesised compound to scavenge $ABTS^{\bullet+}$ [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] radical was determined according to the literature.[41,42] The decrease of absorbance at 734 nm was measured by using a UV-Visible spectrophotometer (1601UV-Shimadzu, Australia). ABTS radical scavenging activity was measured using BHT, ascorbic acid (AA) and catechin (Sigma Chemical Co, USA) as standards; the percentage scavenging was calculated from the formula:

$$\% \text{ Scavenging} = [(OD_{control}-OD_{test})/(OD_{control})x100].$$

In vitro anti-xanthine oxidase (XO) assay

The inhibitory activity of each compound was determined using a slight modification of the reference method.[43] Allopurinol (Sigma–Aldrich, St. Louis, USA) was used as a positive control for the inhibition test. Residual activities were calculated by comparing results to the control without an inhibitor. XO activity was expressed as a percent inhibition of xanthine oxidase, calculated as (1-B/A) x 100, where A is the change in absorbance of the assay without the test samples (Δ abs with enzyme -Δ abs without enzyme), and B is the change in absorbance of the assay with the test sample (Δ abs with enzyme - Δ abs without enzyme).

CONCLUSIONS

Allopurinol is a potent inhibitor of xanthine oxidase which has been widely used to treat gout and hyperuricaemia. However, due to the unwanted side effects of allopurinol, an alternative treatment with increased therapeutic activity and fewer side effects is necessary. Therefore, the new pyrazole and pyrazoline derivatives were synthesised. All of new compounds were tested *in vitro* for the XO inhibitory and antioxidant activity. In this study, pyrazoline derivative compounds were found to be effective antioxidants in several *in vitro* assays including: $ABTS^{\bullet+}$, $DPPH^{\bullet}$ radical scavenging and CUPRAC methods. Compounds which are also the most effective for xanthine oxidase inhibition under *in vitro* conditions were pyrazole derivatives. Moreover, this study will provide an innovation for further studies related to antioxidant properties and potential XO inhibitors of new pyrazole and pyrazoline derivatives.

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