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Design, synthesis and biological evaluation of new 2-aminothiazole scaffolds as phosphodiesterase type 5 regulators and COX-1/COX-2 inhibitors†

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A new series of 2-aminothiazole derivatives was designed and prepared as phosphodiesterase type 5 (PDE5) regulators and COX-1/COX-2 inhibitors. The screening of the synthesized compounds for PDE5 activity was carried out using sildenafil as a reference drug. Strikingly, compounds **23a** and **23c** were found to have a complete inhibitory effect on PDE5 (100%) at 10 μM without causing hypotension and the limited side effect of PDE5 inhibitors, suggest a distinctive therapeutic role of these derivatives in erectile dysfunction. On the other hand, compounds **5a**, **17**, **21** and **23b** increased the PDE5 activity (PDE5 enhancers) at 10 μM . In addition, the study includes the screening of the COX-1/COX-2 inhibition induced by the synthesized compounds. All tested compounds have an inhibitory effect against COX-1 activity (IC_{50} = 1.00–6.34 μM range) and COX-2 activity (IC_{50} = 0.09–0.71 μM range). Moreover, a molecular docking study was implemented to reveal the binding interactions of potent compounds in the binding sites of PDE5 (PDB ID 2H42), COX-1 and COX-2 (PDB ID 3LN1) enzymes. For the interaction with the PDE5 enzyme, activator compounds had a strong binding mode (HB with Gln817:A) than inhibitory derivatives. Both types of compounds are considered as PDE5 regulators. This novel finding will encourage us to discover a new pharmacological application of small chemical entities as the PDE5 enhancer, or will lower side effects as PDE5 inhibitors. All active compounds adopted the Y-shape along the COX-2 active site.

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1. Introduction

Phosphodiesterase 5 inhibitors and the COX activity have recently emerged and the phosphodiesterase type 5 inhibitors (PDE5-Is) have been reported as potential therapy for neuroinflammatory and neurodegenerative diseases.¹ PDE5-Is produce an anti-inflammatory and neuroprotective action by enhancing the expression of nitric oxide synthases and in the accumulation of cGMP, in addition to activating protein kinase G (PKG), which play an important role in the development of several neurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD), and multiple sclerosis (MS).² Moreover, clinical experiments indicate

that chronic inflammation is the main factor in benign prostatic hyperplasia (BPH) progression. The use of anti-inflammatory agents such as steroids and cyclooxygenase-2 (COX-2) inhibitors have been reported as treatment for prostate diseases.³ It has been reported that PDE5 inhibitors (PDE5-Is) relax the smooth muscle fibers of the bladder and prostate by NO/cGMPc signaling, and then reduce the hyperactivity of the autonomic nervous system.⁴ As a result, we can say that phosphodiesterase-5 inhibitors can be used to treat prostatic inflammation. The literature survey revealed that sildenafil (Fig. 1) (as a PDE5 inhibitor) has been shown to be beneficial in the early phases of inflammation and vascular remodeling in a pulmonary arterial hypertension (PAH). The administration of sildenafil significantly reduced the inflammation in the acute stage of the disease and prevented pulmonary arterial remodeling. These results revealed that sildenafil has a direct anti-inflammatory effect, in addition to its vasodilatation and anti-proliferative effects.⁵ The anti-inflammatory effect of PDE5 inhibitors was confirmed by another study in the rat model of colitis.⁶ On the other hand, PDE activators (biochemicals that activate PDE) have many applications in biochemical and physiological research. For example, calmodulin (a multifunctional intermediate calcium-binding messenger protein expressed in all eukaryotic cells) is an activator of NOS, PDE and CaMK.⁷ Another

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example of PDE activators is UCR1C, a novel activator of PDE4 long isoforms, where PDE4 activation by UCR1C attenuates cardiomyocyte hypertrophy.⁸ Regulatory molecules are chemical entities that either increase or inhibit enzyme activity. Compounds that enhance the activity of an enzyme are called activators, while those that decrease the activity of an enzyme are called inhibitors.

Different modifications in sildenafil pharmacophoric features were performed in our previous studies for the design of new pyrazolo[3,4-*d*]pyrimidin-4-(5*H*)-one derivatives as inhibitors of phosphodiesterase enzyme type 5 (PDE5) as sildenafil analogues.^{9,10} Most of the explored tested compounds showed the same binding mechanism as vardenafil, and some of these compounds showed promising activity in comparison to sildenafil in the pharmacological studies (Fig. 1). The results indicated that compounds with a styryl moiety or those with a longer chain alkyl group are the most active derivatives.^{9,10}

Based on these biological interests, in addition to our successful synthesis of fused moieties using 3-oxobutanamide derivatives as starting materials,^{11–14} it was of interest to prepare a series of 2-aminothiazole derivatives (**2**, **5a,b**, **7**, **11**, **13**, **16–19**, **22**, **23a–c**) using 3-oxobutanamides as a precursor. We investigated their PDE5 regulatory activity, in addition to the COX-1 and COX-2 inhibitory activity (Fig. 2). Furthermore, a docking study was carried out for all synthesized compounds to predict their binding mode with the PDE5 compared to sildenafil and avanafil (Fig. 1) as standard PDE5 inhibitors. In the case of COX-1 and COX-2, the binding mode of the compounds was compared to celecoxib as the COX inhibitor (Fig. 1).

In order to find a new drug candidate with selective COX-2 inhibition and PDE5 inhibitors, a comparative shape alignment was carried out to synthesize a new lead candidate. Regarding PDE5 inhibition, the comparative shape alignment between sildenafil and avanafil with enamide **23c** showed that the replacement of the methyl group with an aryl moiety is

suggested. In the comparison of **23b** with celecoxib (also illustrated), the replacement of methyl group with aryl moiety is suggested.

Finally, we hypothesize the synthesis of new derivatives of aminothiazole, especially with the general formula in Fig. 2.

2. Results and discussion

2.1. Chemistry

As we previously reported,^{11–14} 3-oxobutanamide can be utilized as a precursor in the synthesis of several heterocyclic substances. In the current work, we explored the reaction of 3-oxobutanamides **1** with some electrophilic and nucleophilic reagents. Thus, the treatment of **1** with sodium nitrite in acetic acid at 0 °C afforded the 2-hydroxyimino-3-oxo-*N*-thiazole-2-yl-butylamide **2**.¹⁵ The structure of compound **2** was confirmed by spectroscopic data. Moreover, the reduction of compound **2** with zinc dust in acetic acid furnished the intermediate 2-amino-3-oxo-*N*-thiazole-2-yl-butylamide **3** (not isolated), which converted directly to the pyrrole derivatives **5a,b** on treatment with an equimolar amount of ethyl acetoacetate and acetyl acetone, respectively, through the intermediate **4**.¹⁵ Compound **5** may proceed by condensation of the carbonyl group in active methylene to amino function in compound **3** to form the Schiff's base intermediate **4**, which under intramolecular cyclization by loss of water, forms the final product **5a,b** (Scheme 1).

Similarly, when compound **1** is allowed to react with hydroxylamine hydrochloride in refluxing ethanol in the presence of sodium acetate, the pyrazole derivative **7** was obtained through the unisolated oxime **6**. The synthetic potential of **1** toward a variety of nucleophilic reagents was also investigated. Thus, the boiling of compound **1** in sodium ethoxide solution following the addition of an equimolar amount of ethyl

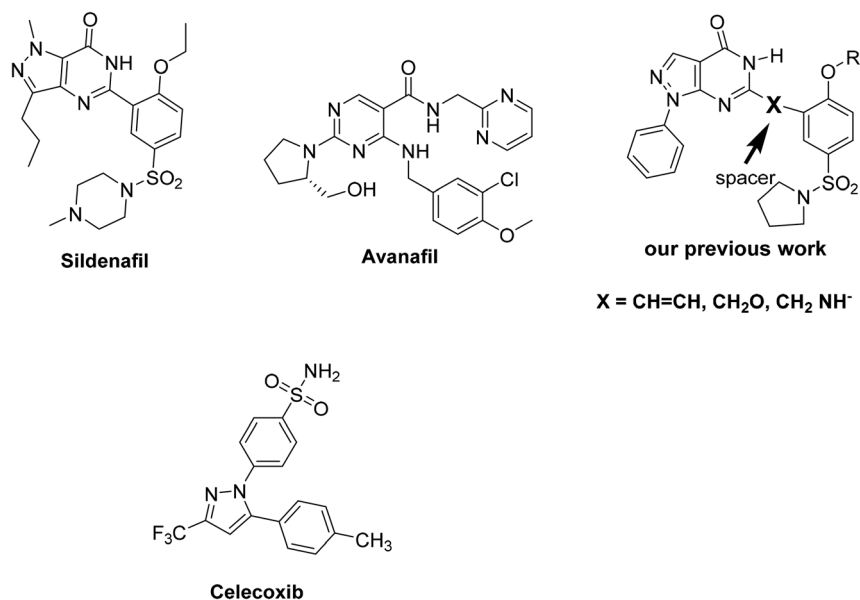


Fig. 1 Structures of PDE5 inhibitors and COX-2 inhibitory reference drugs.



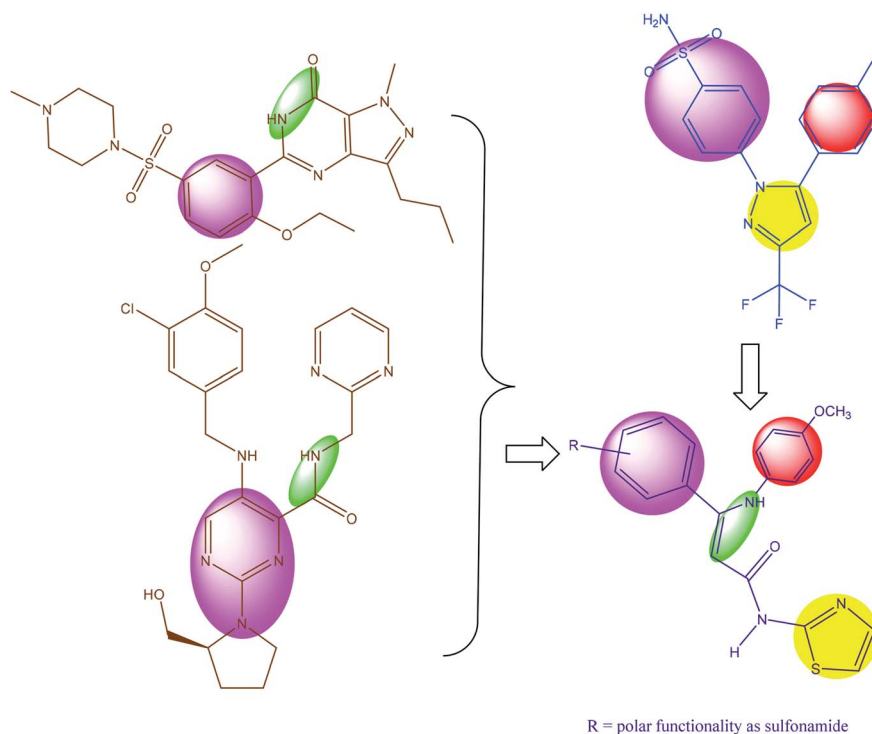
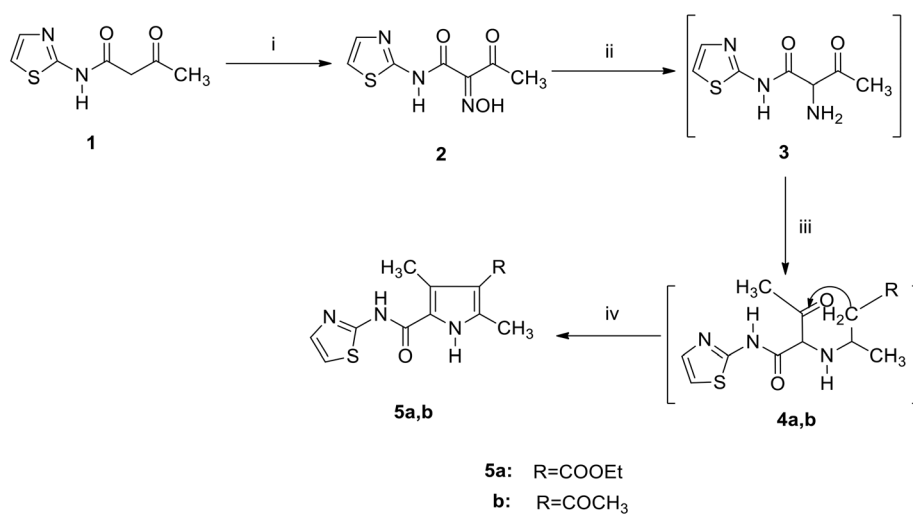


Fig. 2 Rational for the design of more potent PDE5 inhibitors and selective COX-2.

chloroacetate did not yield the expected **9**, and only ethyl 4-(thiazol-2-ylamino) butanoate **11** was obtained.¹⁶ Compound **11** is assumed to proceed by condensation of ethyl chloroacetate to the sodium salt of compound **1**, forming the (not isolated) intermediate **10** by loss of a HCl molecule and subsequent loss of an acetaldehyde molecule to form **11**. Compound **11** was confirmed based on its compatible spectroscopic data (IR, ¹H NMR, ¹H, ¹H COSY, ¹³C NMR, DEPT-135 of ¹³C NMR, NOE of ¹³C NMR). ¹H NMR revealed a triplet signal at $\delta = 1.23$ ppm, $J =$

7.2 Hz assigned to CH₃, triplet signals at $\delta = 2.78$ ppm, $J = 6.4$ Hz, $\delta = 2.87$ ppm, $J = 6.4$ Hz assigned for 2CH₂, quartet signal at $\delta = 4.13$ ppm, $J = 7.2$ Hz assigned to CH₂ ester, doublet signals at $\delta = 6.96$ ppm, $\delta = 7.47$ ppm assigned to 2 thiazole H-4, H-5, and a singlet signal at $\delta = 11.12$ ppm for the NH group. In the ¹H, ¹H COSY spectrum for compound **11**, triplet protons for the methyl group at $\delta = 1.23$ ppm showed J^3 correlation with quartet methylene protons at $\delta = 4.13$ ppm to indicate an ethyl moiety. At the same time, the methylene protons at $\delta =$

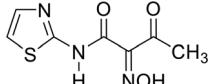
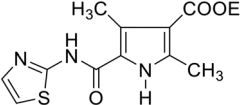
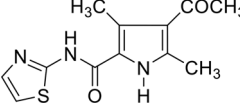
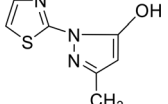
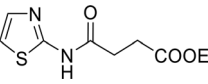
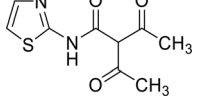
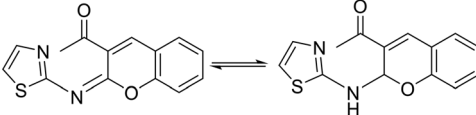
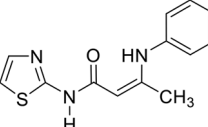
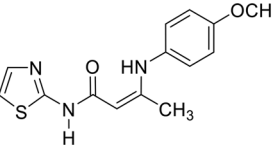
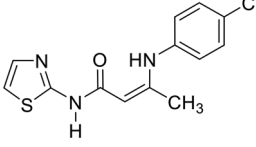


Reagents: (i) NaNO₂ / AcOH; (ii) Zn / AcOH; (iii) CH₃COCH₂R / AcOH; (iv) -H₂O

Scheme 1



Table 1 Effect of tested compounds on PDE5 (10 μ M) and MABP, and consensus scores against PDE5^{a,b,c}

Comp. no.	Comp. structure	PDE5 activity (consensus scores)	MABP
2		53 \downarrow (122)	115 ^{###}
5a		222 \uparrow (28)	108 ^{###}
5b		15 \uparrow (49)	98
7		75 \downarrow (126)	81 [#]
11		3.5 \uparrow (100)	85 ^{&}
17		371 \uparrow (110)	87 ^{&}
21		4290 \uparrow	118 ^{###}
23a		100 \downarrow (57)	108
23b		15 \uparrow (50)	110 ^{###}
23c		100 \downarrow (48)	100
Control (DMSO)	—	—	93.10 \pm 1.55
Nitroprusside	—	—	52.10 \pm 1.11 ^{###}
Sildenafil	Fig. 1	100 \downarrow	72.50 \pm 2.9 ^{###}

^a Data were expressed as means \pm SE ($n = 4$). ^b Multiple comparisons were done using the one way ANOVA, followed by Tukey–Kramer as the post-ANOVA test. ^c #: Significantly different from the control group at $P < 0.05$. &: Significantly different from the sildenafil group at $P < 0.05$.

ester **13** rather than the expected 3-oxo-2-(thiazole-2-yl-carbamoyl)-butyric acid ethyl ester **12**. Compound **13** was established on the basis of the spectroscopic data. ¹H NMR revealed a triplet signal at $\delta = 1.37$ ppm, $J = 6.8$ Hz assigned to

CH₃, quartet signal at $\delta = 4.30$ ppm, $J = 6.8$ Hz, doublet signal at $\delta = 6.91$ ppm, $\delta = 7.39$ ppm assigned to 2 thiazole-H, and a singlet signal at $\delta = 12.81$ ppm assigned to the NH group. ¹H, ¹H COSY showed a strong J^3 coupling between the triplet



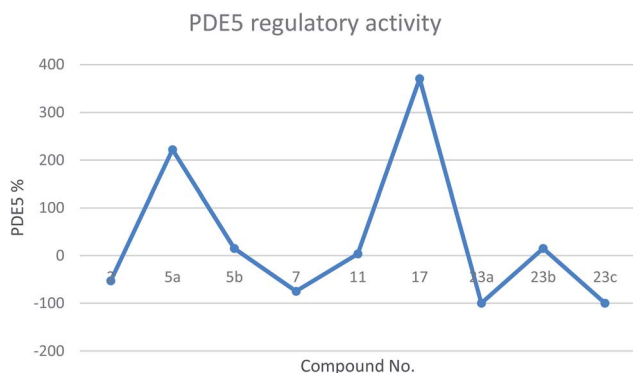


Fig. 3 Line chart representing the PDE5 regulatory activity (either increase or decrease in PDE5%) of the tested compounds.

methyl protons at $\delta = 1.36$ ppm and quartet methylene protons at $\delta = 4.30$ ppm to confirm the ethyl group. ^{13}C NMR showed signals at $\delta = 14.47$ ppm (q), $\delta = 62.13$ ppm (t), $\delta = 112.33$ ppm (d), $\delta = 136.71$ ppm (d), $\delta = 154.02$ ppm (s), and $\delta = 161.94$ ppm (C=O) group. (DEPT-135) of ^{13}C NMR recorded the appearance of only the sp^3 and sp^2 carbons at $\delta = (+)14.46$ ppm (q), $\delta = (-)62.13$ ppm (t), $\delta = (+)112.31$ ppm (d), and $\delta = (+)136.70$ ppm (d). The NOE (Nuclear Overhauser Effect) of ^{13}C NMR spectra showed two doublet aromatic methene carbons at $\delta = 111.31$ – 113.18 ppm and $\delta = 135.74$ – 137.60 ppm. The coupling constant difference indicates that they are geminal protons. At the same time, the triplet methylene carbon at $\delta = 60.66$ – 63.61 ppm and quartet methyl carbon at $\delta = 12.57$ – 16.37 ppm were observed and the quaternary carbon at $\delta = 154.02$ ppm (s), and at $\delta = 162.05$ (s) for the (C=O) group. Structural elucidation of compound **13** came from its synthesis by another method, conducting the believes 2-aminothiazole and ethyl chloroformate in boiling solution of ethanolic sodium ethoxide (mp; mx.mp and TLC). We suggest that compound **13** proceeds by the addition of ethyl chloroformate to **8** (the unisolated sodium salt of compound **1**). The structure was broken and freed from

Table 2 *In vitro* COX-1 and COX-2 inhibitory activity of the target compounds and reference drugs^{a,b}

Comp. no.	COX-1 IC_{50}^a (μM)	COX-2 IC_{50}^a (μM)	COX-2 S.I. ^b
2	1.5	0.11	13.64
5a	1.6	0.10	16
5b	1.3	0.24	5.4
7	6.34	0.63	10.06
11	1.7	0.21	8.1
17	1.4	0.09	15.56
21	1.0	0.33	3.03
23a	6.30	0.71	8.87
23b	5.74	0.56	10.25
23c	6.33	0.61	10.38
Celecoxib	7.21	0.83	8.68

^a The concentration of the test compound that produces 50% inhibition of COX-1, COX-2 enzyme activity. The result is a mean of two values obtained by the assay of enzyme kits. ^b The *in vitro* COX-2 selectivity index (COX-1/COX-2).

2-amino-thiazole, which react directly with ethylchloroformate present in the reaction medium to give compound **13** (Scheme 2).

On the other hand, a reaction of compound **1** with α -chloroacetylchloride afforded the pyrrole derivative **16** through the intermediates **14** and **15**. In the same manner, the reaction of compound **1** with acetyl chloride in benzene under refluxing gave the adduct **17**. Compound **17** was confirmed by its reaction with nucleophilic reagents (such as hydrazine hydrate) and urea to obtain some azole and azine derivatives. So, the treatment of compound **17** with hydrazine hydrate in ethanol under reflux afforded the corresponding pyrazole derivative **18**. In the same way, the pyrimidine derivative **19** was synthesized by reaction of compound **17** and urea in ethanol under reflux (Scheme 3).

In contrast, the behavior of compound **1** with 2-hydroxy benzaldehyde in pyridine solution under reflux gave the benzopyrane derivative **21** through the intermediate **20**. However, under the reaction conditions, the quinoline derivative **22** was obtained through a Dimroth rearrangement of **21**. On the other hand, the reaction of compound **1** with aromatic amines was also investigated. So, it reacts with aromatic amines (like aniline, *p*-anisidine and *p*-chloroaniline) to give the adducts **23a–c** with the loss of water. Structures **23a–c** were confirmed on the basis of spectroscopic data. In the ^1H , ^1H COSY spectrum, the protons A–B system showed a strong J^3 coupling. At the same time, a doublet proton H4 at $\delta = 6.86$ ppm showed a strong J^3 correlation with a doublet proton at $\delta = 7.37$ ppm for the thiazole ring (Scheme 4).

2. 2. Pharmacological evaluation

2. 2. 1 Phosphodiesterase 5 interaction and antihypertensive activity. The vasorelaxant effects of the tested compounds are presented in Table 1. The data showed that the MABP values of compounds **2**, **5a**, **21** and **23b** are significantly higher than those of the control, while the values of compounds **5b**, **23a** and **23c** are higher than the control values in a nonsignificant manner. In addition, the values of compounds **11** and **17** are significantly higher than the sildenafil values, but insignificantly lower than the control values. In contrast, the value of compound **7** is significantly lower than that of the control, and insignificantly higher than the sildenafil one. In addition, the

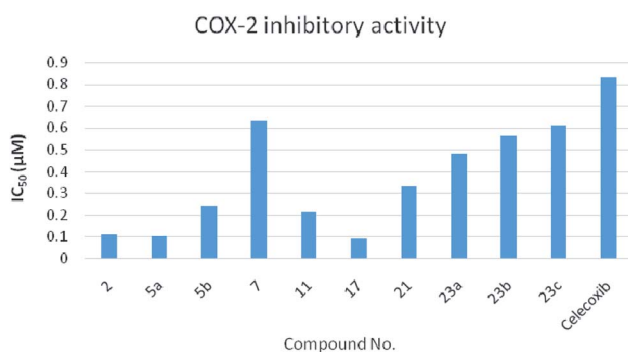


Fig. 4 Chart representing the COX-2 inhibitory activity of the tested compounds against celecoxib as a reference.



results of the PDE5 regulatory activity of the tested compounds against sildenafil are summarized in Table 1, and graphically presented in Fig. 3. The compounds are categorized as three groups based on their action against PDE5: (a) activators, which are compound **21** (PDE5 4290% enhancement), compound **17** (PDE5 371% enhancement), and compound **5a** (PDE5 222% enhancement); (b) weak regulators (activators), which are compounds **5b** and **23b** (PDE5 15% enhancement), and compound **11** (PDE5 3.5% enhancement); and finally (c) inhibitors, which are compounds **23a** and **23b** (PDE5 100% inhibition), compound **7** (PDE5 75% inhibition), and compound **2** (PDE5 53% inhibition).

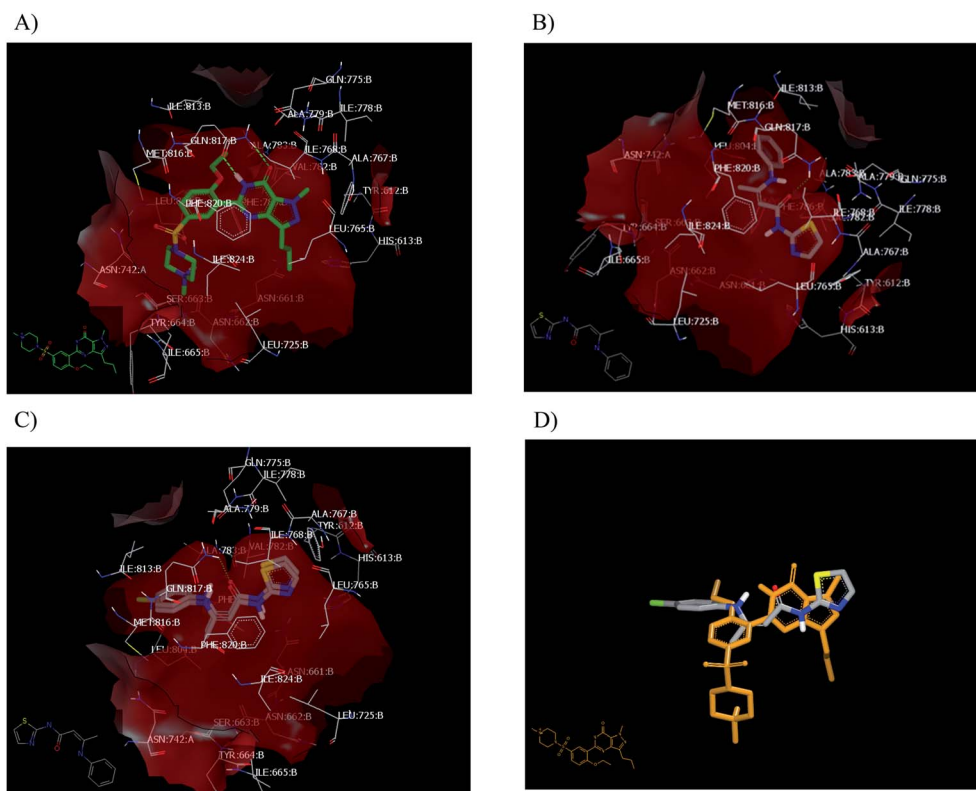
The mean arterial blood pressure (MABP) results and that of PDE5 revealed that compounds **23a** and **23c** have a good chance to be used in erectile dysfunction without the development of the common side effect, hypotension, that is associated with the use of such drugs to manage erectile dysfunction. In contrast, compounds **5a**, **7**, and **21** illustrated the enhancement in PDE5 activity (Table 1). Other compounds, such as **5b**, **11**, **23b**, showed very low activity (either in inhibition or enhancements). In a comparison study between compounds **5a** and **5b** or **23a** and **23c** to **23b**, it is clear that any change in pharmacophoric feature in such types of compounds has a great effect on the PDE5 activity. Notably, compound **21** has a remarkable effect on PDE5 as a strong activator. This behavior could be attributed to the presence of a fused heterocyclic system (chromene ring) linked with the 2-aminothiazole moiety, and the capability to form an amine-

imine tautomerism. This, of course, will increase the compound binding with the specific amino acid clefts inside the active site. We speculate that, according to the strength of the compound interaction with PDE5, a conformational change of the enzyme will occur. Therefore, the activity fluctuated from enhancement to inhibition.

2. 2. 2 In vitro anti-inflammatory activity. The prepared compounds were assayed against COX-1 and COX-2 using a colorimetric enzyme immunoassay (EIA) kit (Cayman Chemicals Inc., Ann Arbor, MI, USA) to screen the isozyme-specific inhibition. The potency of the testing compounds was estimated as the concentration causing 50% enzyme inhibition (IC_{50}). The *in vitro* selective index (SI) COX-1/COX-2 isozyme inhibition studies determined the ability of all synthesized compounds in inhibiting ovine COX-1 and human recombinant COX-2.¹⁷

The data (Table 2) showed that the target compounds have higher inhibitory activities against both COX-1 isozyme (IC_{50} = 1.00–6.34 μ M range) and COX-2 isozyme (IC_{50} = 0.09–0.71 μ M range), with COX-2 selectivity indexes in the range of 3.03 to 16 in comparison with the COX-2 selective reference drug celecoxib (COX-1 IC_{50} = 7.21 μ M, COX-2 IC_{50} = 0.83 μ M and S.I. = 8.68). In addition, the COX-2 inhibitory activity of the tested compounds against celecoxib is graphically represented in Fig. 4.

In the comparison between the compound activities on PDE5 and anti-inflammatory effect, all compounds have COX-2 selectivity whether they are an activator or inhibitor to PDE5.



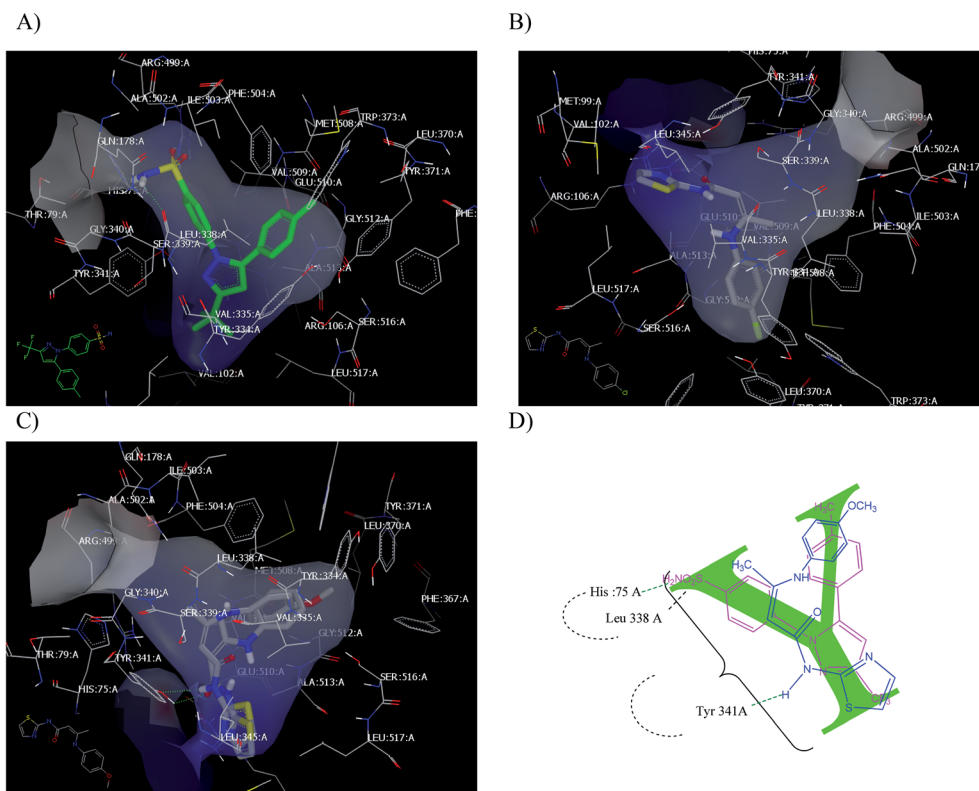


Fig. 7 (A) Celecoxib with the COX-2 active site (PD: ID 3IN1); (B) enamide **23c**; (C) **23a** and **23b** overlay; (D) Y shape of enamide (blue) inside the receptor overlay with celecoxib (pink colour).

the receptor in which the nitrogen atom of the thiazole moiety is oriented toward Gln: 817, as shown in Fig. 6C. This behavior differs from the other enamide **23a**. Both compounds **5a** and **21** occupied the receptor domain with high similarity in mode and pose, as shown in Fig. 6D.

2. 3. 2 COX-2 inhibition. The docking started point was determined by examining the interaction of the standard drug celecoxib with the COX-2 enzyme. Celecoxib was found to interact with the receptor of PDB ID: 3LN1,²¹ taking its known pose with the Y shape (pyrazole core with vicinal diaryl pharmacophores), as shown in Fig. 7A, with a best consensus score of 0.00.

All examined compounds interacted with the COX-2 enzyme and docked inside the receptor. Some of these compounds exhibited binding poses that were similar to that of celecoxib. Enamide **23c** has the highest consensus score value (12) with the formation of the hydrophobic–hydrophobic interaction, as shown in Fig. 7B. Enamides **23a** and **23b** with consensus scores of 19 and 14, respectively, adopted binding poses with the formation of HB with Tyr 341:A, as shown in Fig. 7C. From a medicinal chemistry perspective, the enamides adopted the Y shape inside the receptor, as illustrated in Fig. 7D. Enamide **23b** and celecoxib interacted through HBs with the amino acids cleft of Tyr 341:A, Leu 338:A, and Hist 75:A.

3. Conclusion

New aminothiazole derivatives having different functionalities were synthesized and their activity with PDE5 and COX-1/COX-2

was examined. The synthesized compounds adopted two different patterns against PDE5. Compounds **23a** and **23b** exhibited 100% inhibition of PDE5 at 10 μ M without a notable decrease in MABP. This kind of compound has the benefit over current drugs and PDE5 inhibitors, which have a hypotensive effect as a drawback. In contrast, compounds **5a**, **21**, **17** and **23b** increased the PDE5 activity (PDE5 enhancers) at 10 μ M. Both types of compounds are considered PDE5 regulators. To the best of our knowledge, this is the first report of small molecules as PDE5 enhancers with the hope of finding a pharmacological application. All compounds showed anti-inflammatory activity based on the COX-1/COX-2 IC_{50} % values.

All tested compounds have an inhibitory effect against the COX-1 activity (IC_{50} = 1.00–6.34 μ M range) and COX-2 activity (IC_{50} = 0.09–0.71 μ M range). The selectivity indexes of these compounds to the COX-2 isoenzyme fall in the range of 3.03 to 16 in comparison with celecoxib, a selective COX-2 inhibitor (COX-1 IC_{50} = 7.21 μ M, COX-2 IC_{50} = 0.83 μ M and S.I. = 8.68).

We envisioned that there is a relationship between the strength of the compound interaction and their effect on the enzyme from inhibition, ranging from nothing to activation.

4. Experimental

4.1. Chemistry

Melting points were uncorrected and determined on a Stuart melting point apparatus (Stuart Scientific, Redhill, UK).



Elemental analyses of C, H and N were performed on a Perkin-Elmer 2400 analyzer (Perkin-Elmer, Norwalk, CT, USA). The IR spectra (KBr) were measured on a Shimadzu IR 435 spectrophotometer and the values are represented in cm^{-1} . ^1H NMR (400 MHz) and ^{13}C NMR (75 MHz) spectra were carried out using a Varian Gemini 300-BB spectrophotometer (Bruker, Munich, Germany). Splitting patterns were designated as follows: s: singlet; d: doublet; t: triplet; m: multiplet, and chemical shift values were recorded in ppm on a scale. Mass spectra were run on a Shimadzu Qp-2010 plus. The progress of the reactions was monitored by TLC using TLC sheets pre-coated with UV fluorescent silica gel (Merck 60 F 254), and was visualized using a UV lamp. The chemicals used were supplied from Acros (New Jersey, USA).

4.1.1 Preparation of 2-(hydroxyimino)-3-oxo-N-(thiazol-2-yl)butanamide (2). A mixture of 3-oxobutanamides **1** (0.03 mol) in 100 ml of glacial acetic acid was cooled in an efficient freezing mixture to 5°C , and a cold solution of (0.0147 mole) sodium nitrite in 5 ml water was added dropwise with vigorous stirring at such a rate that the temperature remained between $5\text{--}7^\circ\text{C}$. With efficient cooling, about one-half hour was required to add the nitrite. The mixture was stirred for one-half hour longer, and then allowed to stand for 4 h. During this time, it warmed up to room temperature. Finally, the solid product formed was collected by filtration and recrystallized from aqua's ethanol to give **2**.

Brown crystals; yield, 65%; mp 182°C ; ^1H NMR (DMSO- d_6): δ 2.40 (s, 3H, CH_3), 7.28 (d, 1H, CH-thiazole, $J = 0.8$ Hz), 7.50 (d, 1H, CH-thiazole, $J = 1.6$ Hz), 12.54 (s, 1H, NH), 13.06 (s, 1H, N-OH); ^{13}C NMR (DMSO- d_6): δ 25.28, 114.17, 137.85, 152.16, 156.77, 160.18, 194.35; Dept 135 NMR: δ 25.22, 114.10, 137.85; MS (relative intensity) m/z : 214.11 (M^+ , 7.8%); anal. calcd for $\text{C}_7\text{H}_7\text{N}_3\text{O}_3\text{S}$ (213.21): C, 39.43; H, 3.31; N, 19.71; S, 15.04%; found: C, 39.41; H, 3.33; N, 19.72; S, 15.05%.

4.1.2 General procedure for the synthesis of compounds 5a,b. To a solution of **2** (0.01 mol) in 50 ml of glacial acetic acid, portions of (0.5 gram atoms) of zinc dust were added quickly. It was stirred until the liquid boiled, and then frequently enough to keep it boiling. 0.01 mol of ethyl acetoacetate and acetyl acetone were added. After the addition was completed, the mixture was heated by a burner and refluxed for one hour. While still hot, the contents of the flask were decanted from the remaining zinc into a beaker containing 500 ml of water, which was vigorously stirred. The zinc residue was washed with two 50 ml portions of hot glacial acetic acid, which were also decanted into the water. After standing overnight, the crude product was filtered by suction, washed on the filter with two 500 ml portions of water, and dried in air to a constant weight to give **5a,b**.

4.1.3 Ethyl 2,4-dimethyl-5-(thiazol-2-ylcarbamoyl)-1H-pyrrole-3-carboxylate 5a. Dark red crystals from aqua's ethanol; yield 45%; mp 147°C ; IR (KBr) ν $\text{cm}^{-1} = 3450$ (2NH), 3081 (CH arom.), 2921 (CH aliph.), 1657 (C=O); ^1H NMR (CDCl_3 - d_1): δ 1.33 (t, 3H, CH_3 , $J = 7.2$ Hz), 2.58 (s, 3H, CH_3), 2.69 (s, 3H, CH_3), 4.24 (q, 2H, CH_2 , $J = 7.2$ Hz), 7.07 (d, 1H, CH-thiazole, $J = 4.4$ Hz), 7.51 (d, 1H, CH-thiazole, $J = 4.4$ Hz), 11.12 (s, 2H, NH); ^{13}C NMR (CDCl_3 - d_1): δ 12.17, 14.36, 14.75, 59.77, 113.17, 114.81,

117.12, 125.34, 137.30, 142.41, 156.39, 162.86, 164.83; Dept 135 NMR δ 12.17, 14.36, 14.75, 59.81, 113.15, 125.34; anal. calcd for $\text{C}_{13}\text{H}_{15}\text{N}_3\text{O}_3\text{S}$ (293.34): C, 53.23; H, 5.15; N, 14.32; S, 10.93%; found: C, 53.25; H, 5.12; N, 14.30; S, 10.91%.

4.1.4 4-Acetyl-3,5-dimethyl-N-(thiazol-2-yl)-1H-pyrrole-2-carboxamide 5b. Brown crystals from aqua's ethanol; yield 56%; mp 220°C ; IR (KBr) ν $\text{cm}^{-1} = 3404$ (NH), 3086 (CH arom.), 2918 (CH aliph.); 1687 (C=O); ^1H NMR (DMSO- d_6): δ 2.39 (s, 3H, CH_3), 2.51 (s, 3H, CH_3), 2.57 (s, 3H, CH_3), 7.22 (d, 1H, CH-thiazole, $J = 3.6$ Hz), 7.51 (d, 1H, CH-thiazole, $J = 3.6$ Hz), 11.79 (s, 2H, 2NH); ^{13}C NMR (DMSO- d_6): δ 12.43, 14.79, 31.21, 113.30, 119.01, 122.68, 129.03, 137.14, 137.88, 158.56, 194.40; MS (relative intensity) m/z : 265.9 (M^{2+} , 93.3%); anal. calcd for $\text{C}_{12}\text{H}_{13}\text{N}_3\text{O}_2\text{S}$ (263.32): C, 54.74; H, 4.98; N, 15.96; S, 12.18%; found: C, 54.76; H, 4.95; N, 15.94; S, 12.16%.

4.1.5 Preparation of 3-methyl-1-(thiazol-2-yl)-1H-pyrazol-5-ol 7. A mixture of **1** (0.01 mol) and hydroxyl amine hydrochloride (0.01 mol) was refluxed in ethanol containing fused sodium acetate for 8 h, and then 30 ml of glacial acetic acid was stirred and portions of (0.5 gram atoms) of zinc dust were added. After the addition was completed, the mixture was heated by a burner and refluxed for one hour. While still hot, the contents of the flask were decanted from the remaining zinc into a beaker containing 500 ml water, which was vigorously stirred. The zinc residue was washed with two 50 ml portions of hot glacial acetic acid, which were also decanted into the water. After standing overnight, the crude product was filtered by suction, washed on the filter with two 500 ml portions of water, and dried in air to a constant weight, and then left to cool. The solid product formed was filtered off and recrystallized from aqua's ethanol to give **7**.

Dark red crystals; yield: 48%, mp 290°C ; IR (KBr) ν $\text{cm}^{-1} = 3426$ (OH), 3027 (CH arom.), 2949 (CH aliph.); ^1H NMR (DMSO- d_6): δ 2.15 (s, 3H, CH_3), 5.65 (s, 1H, CH pyrazole), 7.05 (d, 1H, CH-thiazole, $J = 1.6$ Hz), 7.38 (d, 1H, CH-thiazole, $J = 1.6$ Hz), 11.77 (br. s, 1H, OH); anal. calcd for $\text{C}_7\text{H}_7\text{N}_3\text{O}_3\text{S}$ (181.21): C, 46.40; H, 3.89; N, 23.19; S, 17.69%; found: C, 46.41; H, 3.88; N, 23.20; S, 17.66%.

4.1.6 Preparation of ethyl 4-oxo-4-(thiazol-2-ylamino)butanoate 11. To a solution of compound **1** (0.01 mol) in 30 ml of absolute ethanol containing 0.01 mol of clean sodium metal, 0.01 mol of ethyl chloroacetate was added slowly. The reaction mixture was refluxed for 6 h and left to cool, then poured onto ice/water containing a few drops of HCl. The formed solid product was collected by filtration and recrystallized from aqua's ethanol to give **11**.

White crystals; yield: 56%, mp = 154°C ; IR (KBr) ν $\text{cm}^{-1} = 3435$ (NH), 3082 (CH arom.), 2922 (CH aliph.), 1726, 1688 (2C=O); ^1H NMR (CDCl_3 - d_1): δ 1.23 (t, 3H, CH_3 , $J = 7.2$ Hz), 2.78 (t, 2H, CH_2 , $J = 6.4$ Hz), 2.87 (t, 2H, CH_2 , $J = 6.4$ Hz), 4.13 (q, 2H, CH_2 , $J = 7.2$ Hz), 6.96 (d, 1H, CH-thiazole, $J = 3.2$ Hz), 7.47 (d, 1H, CH-thiazole, $J = 4$ Hz), 11.12 (s, 1H, NH); ^{13}C NMR (CDCl_3 - d_1): δ 14.17, 28.84, 30.74, 60.95, 113.50, 136.36, 159.95, 169.64, 172.39; Dept 135 NMR δ 14.11, 28.77, 30.66, 60.91, 113.45, 136.30; NOE NMR δ 12.17-16.04 (q, CH_3), 27.49-31.95 (m, 2 CH_2), 59.49 (t, CH_2), 112.39 (d, CH), 135.63 (d, CH); 159.78 (s), 169.57 (s), 172.32 (s); anal. calcd for $\text{C}_9\text{H}_{12}\text{N}_2\text{O}_3\text{S}$ (228.27): C,



47.35; H, 5.30; N, 12.27; S, 14.05%, found: C, 47.33; H, 5.31; N, 12.29; S, 14.04%.

4.1.7 Preparation of ethyl thiazol-2-ylcarbamate 13. A mixture of 3-oxobutanamide **1** (0.01 mol), ethyl chloroformate (0.01 mol) in sodium ethoxide (20 ml) was refluxed for 12 h, and then left to cool. It was poured onto ice/water, which was vigorously stirred. The solid product formed was collected by filtration and recrystallized from aqua's ethanol to give **13**.

Brown crystals; yield; 58%, mp 142 °C; IR (KBr) ν cm⁻¹ = 3423 (NH), 3080 (CH arom.), 2977 (CH aliph.), 1722, 1644 (2C=O); ¹H NMR (CDCl₃-d₁): δ 1.37 (t, 3H, CH₃, J = 6.8 Hz), 4.30 (q, 2H, CH₂, J = 6.8 Hz), 6.91 (d, 1H, CH-thiazole, J = 3.6 Hz), 7.39 (d, 1H, CH-thiazole, J = 4 Hz), 12.81 (s, 1H, NH); ¹³C NMR (CDCl₃-d₁): δ 14.47, 62.13, 112.33, 136.71, 154.02, 161.94; Dept 135 NMR δ 14.46 (q), 62.13, 112.31, 136.70; NOE NMR δ 12.57 (q, CH₃); 60.66 (t, CH₂); 111.31 (d, CH); 135.74 (d, CH); 154.02 (s); 162.05 (s); anal. calcd for C₆H₈N₂O₂S (172.2): C, 41.85; H, 4.68; N, 16.27; S, 18.62%, found: C, 41.88; H, 4.67; N, 16.29; S, 18.60%.

4.1.8 Preparation of 1-(2,4-dihydroxy-1-(thiazol-2-yl)-1H-pyrrol-3-yl)ethanone 16. A mixture of **1** (0.01 mol), 2-chloroacetyl chloride (0.01 mol) in ethanol (30 ml) was treated with a few drops of piperidine and refluxed for 6 h, and then left to cool. The solid product formed was collected by filtration and recrystallized from ethanol to give **16**.

Brown crystals; yield: 48%, mp 270 °C; ¹H NMR (DMSO-d₆): δ 2.41 (s, 3H, CH₃), 5.001 (s, 2H, 2OH), 6.08 (s, 1H, CH-pyrrole), 7.30 (d, 1H, CH-thiazole, J = 4.8 Hz), 7.76 (d, 1H, CH-thiazole, J = 5.2 Hz); ¹³C NMR (DMSO-d₆): δ 18.18, 109.66, 109.86, 123.37, 146.57, 153.17, 165.13, 167.27, 196.28 (s, C=O); Dept 135 NMR δ 18.04, 109.55, 109.73, 123.24; anal. calcd for C₉H₈N₂O₃S (224.24): C, 48.21; H, 3.60; N, 12.49; S, 14.30%, found: C, 48.22; H, 3.63; N, 12.50; S, 14.31%.

4.1.9 Preparation of 2-acetyl-3-oxo-N-(thiazol-2-yl)butanamide 17. A mixture of (0.01 mol) of magnesium turnings, (0.01 mol) of 3-oxobutanamide **1**, 20 ml of benzene (dried over sodium), and (0.015 mol) of acetyl chloride was heated under reflux for 12 h in a 100 ml round-bottomed flask. This flask was provided with a condenser closed by a calcium chloride tube and supported in an oil bath (85–90 °C). The reaction mixture was cooled in an ice bath, and the liquid portion was decanted into a separating funnel. The residue left in the flask after decantation was washed twice with 10 ml portions of ether, and the ethereal solution was poured over ice. The ether–water mixture was then added to the benzene solution in the separating funnel, and the mixture was shaken thoroughly. The aqueous layer was drawn off and discarded. The benzene–ether solution was washed once with 10 ml of 5% sodium bicarbonate solution, and once with 50 ml of water. Finally, it was dried over calcium chloride. The ether and benzene were removed by distillation from a water bath. The solid product formed was recrystallized from ethanol to give **17**.

Brown crystals; yield: 46%, mp 187 °C; ¹H NMR (DMSO-d₆): δ 2.31 (s, 3H, CH₃), 2.59 (s, 3H, CH₃), 6.43 (s, 1H, CH), 7.29 (d, 1H, CH-thiazole, J = 3.2 Hz), 7.52 (d, 1H, CH-thiazole, J = 3.6 Hz), 13.20 (s, 1H, NH); ¹³C NMR (DMSO-d₆): δ 19.01, 20.09, 113.76, 114.18, 137.54, 161.56, 166.37, 171.82, 177.62; Dept 135

NMR δ 18.99, 20.07, 113.75, 114.16, 137.54; NOE NMR: δ 18.15–20.75, 112.92, 113.19, 136.65, 161.60, 166.41, 171.75, 177.62; anal. calcd for C₉H₁₀N₂O₃S (226.25): C, 47.78; H, 4.45; N, 12.38; S, 14.17%, found: C, 47.76; H, 4.47; N, 12.39; S, 14.15%.

4.1.10 Preparation of 3,5-dimethyl-N-(thiazol-2-yl)-1H-pyrazole-4-carboxamide 18. A mixture of **17** (0.01 mol) and an excess of hydrazine hydrate was refluxed for 6 h, and then left to cool. The solid product formed was collected by filtration and recrystallized from ethanol to give **18**.

Brown crystals; yield: 57%, mp 210 °C; ¹H NMR (DMSO-d₆): δ 2.31 (s, 3H, CH₃), 2.59 (s, 3H, CH₃), 7.29 (d, 1H, CH-thiazole, J = 3.2 Hz), 7.52 (d, 1H, CH-thiazole, J = 3.6 Hz), 9.69 (s, 1H, NH), 12.67 (s, 1H, NH); ¹³C NMR (DMSO-d₆): δ 19.01, 20.09, 114.18, 137.52, 157.14, 161.56, 166.07, 171.82, 177.62; Dept 135 NMR δ 18.99, 20.07, 114.16, 137.59; MS (relative intensity) m/z : 223.23 (M¹⁺, 2.8%); anal. calcd for C₉H₁₀N₄OS (222.27): C, 48.63; H, 4.53; N, 25.21; S, 14.43%, found: C, 48.61; H, 4.50; N, 25.24; S, 14.46%.

4.1.11 Preparation of 4,6-dimethyl-2-oxo-N-(thiazol-2-yl)-1,2-dihydropyrimidine-5-carboxamide 19. A mixture of **17** (0.01 mol) and urea (0.01 mol) in ethanol (30 ml) was refluxed for 6 h, and then left to cool. The solid product formed was collected by filtration and recrystallized from ethanol to give **19**.

Brown crystals; yield: 53%, mp 235 °C; ¹H NMR (DMSO-d₆) (δ ppm); δ 2.07 (s, 3H, CH₃), 2.36 (s, 3H, CH₃), 7.28 (d, 1H, CH-thiazole, J = 0.8 Hz), 7.50 (d, 1H, CH-thiazole, J = 1.6 Hz), 8.76 (s, 1H, NH), 12.81 (s, 1H, NH); ¹³C NMR (DMSO-d₆): δ 17.07, 20.07, 114.25, 137.53, 157.35, 161.52, 166.39, 171.96 (s), 177.48, 196.29; anal. calcd for C₁₀H₁₀N₄O₂S (250.28): C, 47.99; H, 4.03; N, 22.39; S, 12.81%, found: C, 47.96; H, 4.07; N, 22.40; S, 12.78%.

4.1.12 Preparation of 3-acetyl-1-(thiazol-2-yl)quinolin-2(1H)-one 22. A mixture of **1** (0.01 mol) and 2-hydroxy benzaldehyde (0.01 mol) was refluxed for 5 h in pyridine. The solid product formed was collected by filtration and recrystallized from benzene to give **22**.

Yellow crystals; yield: 45%, mp 110 °C; ¹H NMR (CDCl₃-d₁): δ 2.70 (s, 3H, CH₃), 7.30 (d, 1H, CH-thiazole), 7.33 (d, 1H, CH-thiazole), 7.35 (d, 1H, Ar-H), 7.61–7.62 (m, 1H, Ar-H), 7.63–7.65 (m, 1H, Ar-H), 7.65 (s, 1H, Ar-H), 8.49 (s, 1H, CH-quinoline); ¹³C NMR (CDCl₃-d₁): δ 30.74, 114.24, 117.26, 124.83, 129.80, 131.17, 138.57, 139.90, 142.34, 143.97, 156.05, 161.36, 177.05, 198.71; anal. calcd for C₁₄H₁₀N₂O₂S (270.31): C, 62.21; H, 3.73; N, 10.36; S, 11.86%, found: C, 62.24; H, 3.76; N, 10.32; S, 11.83%.

4.1.13 General procedure for the synthesis of compounds 23a–c. In a round-bottomed flask attached to a Dean and Stark constant water separator, which was connected to a reflux condenser, was placed a mixture of 3-oxobutanamide **1** (0.05 mol), 0.05 mol of aromatic amines, 100 ml of benzene, and 1 ml of glacial acetic acid. The flask was heated in an oil bath at about 125 °C, and the water which distilled out of the mixture with the refluxing benzene was removed at intervals. Refluxing was continued until no more water separated, and then for an additional 30 minutes. The benzene was then distilled under reduced pressure. The solid product formed was filtered off and recrystallized from aqua's ethanol to give **23a–c**.



4.1.14 Preparation of (Z)-3-(phenylamino)-N-(thiazol-2-yl)but-2-enamide 23a. Yellow crystals; yield 74%; mp 220 °C; IR cm^{-1} ν = 3192, 3136 (2NH), 3008 (CH arom.), 2914 (CH aliph.), 1644 (C=O); ^1H NMR (DMSO- d_6) (δ ppm): δ 2.03 (s, 3H, CH₃), 5.05 (s, 1H, Ali CH), 7.11 (d, 1H, CH-thiazole, J = 4 Hz), 7.17–7.14 (m, 5H, Ar-H), 7.45 (d, 1H, CH-thiazole, J = 3.6 Hz), 11.03 (s, 1H, NH), 11.65 (s, 1H, NH); ^{13}C NMR (DMSO- d_6) δ 20.20, 87.88, 112.41, 123.60, 124.58, 129.21, 137.50, 138.93, 157.94, 158.55, 166.74; Dept 135 NMR δ 20.15, 87.83, 112.38, 123.55, 124.55, 129.16, 137.47; MS (relative intensity) m/z : 261.46 (M^{2+} , 3.6%); anal. calcd for $\text{C}_{13}\text{H}_{13}\text{N}_3\text{OS}$ (259.33): C, 60.21; H, 5.05; N, 16.20; S, 12.36%, found: C, 60.22; H, 5.07; N, 16.19; S, 12.35%.

4.1.15 Preparation of (Z)-3-((4-methoxyphenyl)amino)-N-(thiazol-2-yl)but-2-enamide 23b. Green crystals; yield 60%; mp = 235 °C; IR (KBr) cm^{-1} ν = 3208, 3124 (2NH), 3011 (CH arom.), 2955 (CH aliph.), 1652 (C=O); ^1H NMR (DMSO- d_6) (δ ppm): δ 1.90 (s, 3H, CH₃), 3.73 (s, 3H, OCH₃), 4.93 (s, 1H, Ali CH), 6.89 (d, 2H, Ar-H, J = 8.8 Hz), 7.04 (d, 1H, CH-thiazole, J = 3.6 Hz), 7.11 (d, 2H, Ar-H, J = 8.8 Hz), 7.38 (d, 1H, CH thiazole, J = 3.6 Hz), 10.38 (s, 1H, NH), 11.51 (s, 1H, NH); ^{13}C NMR (DMSO- d_6): δ 19.95, 55.26, 86.51, 112.26, 114.25, 126.10, 131.63, 137.45, 156.84, 158.91, 166.77; Dept 135 NMR δ 19.90, 55.21, 86.46, 112.21, 114.25, 126.05, 137.40; anal. calcd for $\text{C}_{14}\text{H}_{15}\text{N}_3\text{O}_2\text{S}$ (289.35): C, 58.11; H, 5.53; N, 14.52; S, 11.08%, found: C, 58.13; H, 5.55; N, 14.50; S, 11.09%.

4.1.16 Preparation of (Z)-3-((4-chlorophenyl)amino)-N-(thiazol-2-yl)but-2-enamide 23c. Yellow crystals; yield 68%; mp = 190 °C; IR (KBr) ν cm^{-1} = 3242, 3208 (2N-H), 3056 (CH arom.), 2923 (CH aliph.), 1649 (C=O); ^1H NMR (CDCl_3 - d_1) (δ ppm): δ 1.97 (s, 3H, CH₃), 4.88 (s, 1H, Ali CH), 6.86 (d, 1H, thiazole, J = 2.8 Hz), 6.99 (d, 2H, Ar-H, J = 8 Hz), 7.24 (d, 2H, Ar-H, J = 8 Hz), 7.37 (d, 1H, thiazole, J = 2.8 Hz), 10.86 (s, 1H, NH); 11.96 (s, 1H, NH); ^{13}C NMR (CDCl_3 - d_1) (δ ppm): δ 20.45, 87.83, 112.34, 125.90, 129.24, 131.04, 133.73, 137.34, 159.78, 161.03, 166.36; Dept 135 NMR δ C 20.45, 87.81, 112.34, 126.00, 129.23, 133.62; NOE NMR δ 19.81–22.37 (q), 86.97–88.58 (d), 111.34–113.36 (d), 159.86–161.05 (d), 125.19–137.24 (m, s) 166.31 (s, C=O); anal. calcd for $\text{C}_{13}\text{H}_{12}\text{ClN}_3\text{OS}$ (293.77): C, 53.15; H, 4.12; N, 14.30; Cl, 12.07; S, 10.91%, found: C, 53.11; H, 4.12; N, 14.31; Cl, 12.09; S, 10.90%.

4.2. Pharmacological assessment

4.2.1 Phosphodiesterase 5 enzyme activity assay. The standard enzymatic reaction mixture (total volume of 200 μl) contained 100 ml M Tris-HCl buffer (pH 8.3), 10 mM MgCl_2 , 10 mM KCl at 37 °C. Alfa casein (2 mg) was used as a carrier for protein precipitation when the protein concentration was low. The PDE A1 enzyme sample had a final protein concentration of 0.5 mg ml^{-1} .²² A concentration of 10 μM of the agents under study (sildenafil analogues) was prepared in dimethyl sulfoxide (DMSO) and pre-incubated in the enzymatic mixture for 5 min at room temperature. The reaction was initiated by the addition of the substrate cGMP (5 μM) at 35 °C for 30 min. The reaction was finished by transferring the reaction mixture tubes in a boiling water bath for 3 min. The sample was then centrifuged

and filtered through a nylon-66 filter, 0.2 mm (Rainin Corporation). The clear filtrate obtained may be used directly for HPLC assay or stored at -20 °C. A blank with protein, denatured by boiling water bath for 3 min, with and without substrate was performed. Both incubation time and enzyme concentration were adjusted so that no more than 25% of the substrate was hydrolyzed under the assay conditions. The chromatographic system was an Agilent Technologies 1200 Series, G1315D DAD. The column used was a Zorbax Eclipse. A rapid resolution 4.69×150 mm 3.5 μM particle size. The mobile phase employed for the separation (isocratic elution) consisted of 200 mM ammonium acetate at pH 6.0 with 2% acetonitrile (v/v). The flow rate was 1.5 $\text{cm}^3 \text{min}^{-1}$ and the detector DAD was at 254 nm. The injection volume was 30 mm^3 . Peak identities were confirmed by co-elution with standards, and all assays were done in duplicate.

4.2.2 Arterial blood pressure measurement

4.2.2.1 Experimental animals. Regardless of sex factor, White New Zealand rabbits weighing 2–2.5 kg were obtained from the animal house of the Faculty of Medicine, Assiut University. The animals were housed for one week to adapt to the environmental conditions. Rabbits were fed a standard diet and allowed free access to water. “All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Faculty of Pharmacy, Minia University and approved by the Animal Ethics Committee” of the Faculty of Pharmacy, Minia University.

4.2.2.2 Experimental design. Fifty-two of normotensive white New Zealand rabbits (2–2.5 kg) were selected randomly, and used in this study (four rabbits for each group) to investigate the possible vasorelaxant effect of the selected compounds. Using pentobarbital sodium (30 mg kg^{-1} , i.v.), normotensive rabbits were anesthetized. Then, they were held on their backs with legs fixed and heads pinned down, and the trachea was exposed to cut before proper cannulation was done.

4.2.2.3 Data recoding. After separation, the two muscle bundles of the sternomastoid and the sternothyroid, the common carotid artery lying in the neck between the lateral bundles of muscle (*longus capitis*), and the trachea was exposed and carefully freed from the nerves, veins and connective tissue. The heparinized cannula was carefully inserted and firmly tied *via* fine thread. Then, the cannula was connected to the pressure transducer using a universal oscillograph (Harvard apparatus, ser. no. K10542) for blood pressure recording. The external jugular vein was also tightly cannulated for administration of tested compounds (dissolved in DMSO), followed by heparinized saline (0.90% w/v NaCl).

The mean arterial blood pressure was calculated using the formula:

$$\text{MABP} = \text{DBP} + 1/3(\text{SBP} - \text{DBP}).$$

where MDP = mean arterial blood pressure, DBP = diastolic blood pressure, SBP = systolic blood pressure.

4.2.2.4 Statistical analysis and data interpretation. Statistical analysis was done using GraphPad Prism 5.0 software (Graph-Pad, San Diego, CA, USA). Data were represented as the mean \pm



SE. Multiple comparisons were carried out using the one-way ANOVA with the Tukey–Kramer test as a multiple comparison post-ANOVA test. $P < 0.05$ was selected to refer to statistical significance.

4.2.3 *In vitro* anti-inflammatory activity screening. The *in vitro* COX-1/COX-2 inhibition was detected using an enzyme immunoassay (EIA) kit (Cayman Chemical, Ann Arbor, MI) as the reported procedure for screening the isozyme-specific inhibition. The potency of the tested compounds was determined as the concentration causing 50% inhibition of enzyme activity (IC_{50}). Also, the COX-2 selectivity indexes (S.I. values) were calculated as $IC_{50}(\text{COX-1})/IC_{50}(\text{COX-2})$, and compared with that of the standard drug, celecoxib, which is a selective COX-2 inhibitor.¹⁷

4.3. Docking study

The study was performed according to our previous method using OpenEye software.²³

Conflicts of interest

There are no conflicts to declare.

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