Towards safer anti-inflammatory therapy: synthesis of new thymol–pyrazole hybrids as dual COX-2/5-LOX inhibitors

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ABSTRACT
New thymol – 1,5-disubstituted pyrazole hybrids were synthesised as dual COX-2/5-LOX inhibitors. Compounds 8b, 8g, 8c, and 4a displayed in vitro inhibitory activity against COX-2 (IC50 = 0.043, 0.045, 0.063, and 0.068 μM) nearly equal to celecoxib (IC50 = 0.045 μM) with high SI (316, 268, 204, and 151, respectively) comparable to celecoxib (327). All target compounds, 4a–c and 8a–i, showed in vitro 5-LOX inhibitory activity higher than reference quercetin. Besides, they possessed in vivo inhibition of formalin-induced paw oedema higher than celecoxib. In addition, compounds 4a, 4b, 8b, and 8g showed superior gastrointestinal safety profile (no ulceration) as celecoxib and diclofenac sodium in the population of fasted rats. In conclusion, compounds 4a, 8b, and 8g achieved the target goal. They elicited in vitro dual inhibition of COX-2/5-LOX higher than celecoxib and quercetin, in vivo potent anti-inflammatory activity higher than celecoxib and in vivo superior gastrointestinal safety profile (no ulceration) as celecoxib.

GRAPHICAL ABSTRACT

Introduction
Inflammation includes variety of mechanisms and release of mediators which lead to drawbacks in the cardiovascular system and the renal apparatus. Non-steroidal anti-inflammatory drugs (NSAIDs) are widely used for the treatment of inflammation, pain, fever, and arthritis. NSAIDs decrease prostaglandins production via inhibition of cyclooxygenase (COX) enzymes. Classical NSAIDs inhibit both COX-1 and COX-2 enzymes which lead to anti-inflammatory activity as well as their side effects. Accordingly, several selective COX-2 inhibitors such as celecoxib, valdecoxib, and rofecoxib have been developed and were approved for clinical use due to their low gastrointestinal side effects. However, their long-term use has been reported to cause cardiovascular side effects and was withdrawn from the market. The inhibition of COX-1/COX-2 result in an increased formation of leukotrienes (LTs) through the lipoxygenase (LOX) pathway. Overproduction of LTs induces asthmatic problems, gastric damage, and ulceration. Moreover, 5-LOX has been related to several undesirable physiological effects which are involved in the progression of inflammation, osteoarthritis, and asthma. Accordingly, dual inhibition of COX-2/5-LOX could provide anti-inflammatory effects with reduced side effects. Furthermore, the natural phenol derivative, thymol, was proved to have COX-2 inhibition and anti-inflammatory activities and was found to abolish the activity of 5-LOX in human monocytic (THP-1) cell line. On the other hand, several 1,3-diaryl pyrazoles were reported to have...
remarkable anti-inflammatory activity comparable to the lead celecoxib II. The anti-inflammatory activity of celecoxib remained in the 3-carboxylate derivative III. Moreover, linking phenyl urea to pyrazole in compounds IV and V resulted in dual inhibition of COX-2 and Soluble Epoxide Hydrolase with favourable cardiovascular profile than celecoxib. Furthermore, hybridisation of 1,5-diarylpyrazole with morpholine produced dual COX-2/5-LOX inhibitor with favourable cardiovascular profile than celecoxib. In addition, replacement of morpholine with its bioisostere piperazine and linking it to 4-tert-butylbenzyl moiety in compound VII showed potent inhibition of LT biosynthesis in activated human neutrophils (Figure 1). Moreover, various N-acylhydrazone derivatives were reported to have analgesic, anti-inflammatory, and COX-2 inhibitory activities.

In the present investigation, the COX-2 inhibitor pharmaco-phore, 1,5-diarylpyrazole, was hybridised with thymol, a natural 5-LOX inhibitor, through N-acylhydrazone linker in order to simultaneously inhibit of the key inflammatory enzymes COX-2 and 5-LOX to get compounds having in vivo anti-inflammatory activity with low side effects and better safety profile (Figure 2).

**Experimental**

**Chemistry**

Melting points were determined in open glass capillaries using a Griffin melting point apparatus or an electrothermal capillary tube melting point apparatus and are all uncorrected. Infra-red spectra (IR) were recorded, using KBr discs, by a Perkin-Elmer 1430 Infra-red spectrophotometer in the Central Laboratory, Faculty of Pharmacy, Alexandria University, and Schimadzu FT-IR Affinity-1 Spectrometer in Faculty of Pharmacy, Cairo University. Nuclear magnetic resonance (1H-NMR and 13C-NMR) were determined using Bruker High-Performance Digital FT-NMR Spectrometer Avance III 400 MHz, Faculty of Pharmacy, Cairo University in using deuterated dimethyl-sulphoxide as a solvent. The data were reported as chemical shifts or δ values (ppm) relative to tetramethylsilane (TMS) as internal standard. Signals are indicated by the following abbreviations: s = singlet, d = doublet, t = triplet, q = quartette, and m = multiplet.

Electron impact mass spectra (EIMS) were run on a gas chromatograph/mass spectrophotometer at Al-Azhar University (The Regional Centre of Microbiology and Biotechnology). Relative intensity % corresponding to the most characteristic fragments was recorded. Elemental microanalyses were performed at the microanalytical unit, Al-Azhar University (The Regional Centre of Microbiology and Biotechnology). Reaction progress was monitored by thin-layer chromatography (TLC) on silica gel sheets (60 GF254, Merck, Kenilworth, NJ 07033, USA). The spots were visualised by exposure to iodine vapour or UV-lamp at λ 254 nm for few seconds.

Compounds 1a–c, 2a–c, 3, 5a–c, 6a–g,i, 26 and 7a–g,i were prepared according to reported procedures.

**General procedure for synthesis of N’-(2-Hydroxy-3-isopropyl-6-methylbenzylidene)-3-(substituted phenyl)-1H-pyrazole-5-carboxhydrazides (4a–c).** A mixture of 2-formylthymol 3, appropriate hydrazides 2a–c, and few drops of glacial acetic acid in absolute ethanol was refluxed for 4–6 h, cooled to RT, the precipitate was filtered, washed with diethyl ether and recrystallised from ethanol.

![Figure 1. Structures of compounds having COX-2 or 5-LOX inhibitory activities.](image-url)
1H, CH of C4 of pyrazole; 6.76(d, 1H, J = 7.00 Hz, C5 of thymol); 7.72(d, 1H, J = 7.68 Hz, C2 of isopropyl); 2.38(s, 3H, CH3 of 6-methylthymol); 3.27–3.31 (m, 1H, CH of isopropyl); 6.70(d, 1H, J = 7.60 Hz, C5 of thymol); 7.60(d, 2H, J = 8.40 Hz, C2, C6 of p-methoxyphenyl); 9.07(br.s, 1H, CH of CH=N; D2O exchangeable); 12.64(s, 1H, OH, D2O exchangeable); 13.70(s, 1H, OH of pyrazole, D2O exchangeable); 13C-NMR (100 MHz, DMSO-d6 ppm): 19.12(CH3 of 6-methylthymol); 22.86 (2CH3 of isopropyl); 26.61 (CH of isopropyl); 115.53 (C4 of pyrazole); 116.26 (C1 of thymol); 120.85 (C5 of thymol); 121.65 (C3 of thymol); 125.60 (C4 of thymol); 130.39 (C4 of phenyl); 132.94 (C3 and C5 of phenyl); 133.31 (C2 and C6 of phenyl); 133.79 (C1 of phenyl); 138.35 (C6 of thymol); 141.75 (C3 of pyrazole); 155.25 (C5 of pyrazole); 157.91 (CH of CH=N); 163.67 (C2 of thymol); 164.91 (C = O); EIMS, m/z (relative abundance %): 363.01(M+1) (8.3), 362.17 (M+); 393 (M+); 1H-NMR (400 MHz, DMSO-d6 ppm): 1.19(d, 6H, J = 6.88 Hz, 2CH3 of isopropyl); 2.38(s, 3H, CH3 of 6-methylthymol); 3.27–3.31 (m, 1H, CH of isopropyl); 6.71(d, 1H, J = 7.60 Hz, C5 of thymol); 7.13(d, 1H, J = 7.60 Hz, C4 of thymol); 7.32(s, 1H, CH of C4 of pyrazole); 7.70(d, 2H, J = 7.68 Hz, C3, C5 of bromophenyl); 7.82(d, 2H, J = 7.68 Hz, C2, C6 of bromophenyl); 9.06(br.s, 1H, CH of CH=N; D2O exchangeable); 12.71(s, 1H, OH, D2O exchangeable); 13.93(br.s, 1H of NH of pyrazole, D2O exchangeable); Analysis Calcd for C21H21BrN4O2 (441.33): C, 57.15; H, 4.80; N, 12.70. Found: C, 56.71; H, 4.64; N, 12.85.

**General procedure for synthesis of Methyl 5-(4-substitutedphenyl)-1-(4-substitutedphenyl)-1H-pyrazole-3-carboxylates (6a–i)**: A mixture of sodium salt of diketone 1a–c (1 mol) and substituted

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**Figure 2.** Design of dual COX-2/S-LOX inhibitors.
phenylhydrazine hydrochloride 5a-c (1 mol) in glacial acetic acid (30 ml) was refluxed for 24 h, then cooled to RT and poured on crushed ice, filtered, washed with water, air-dried and then washed with diethyl ether and recrystallised from methanol.

**Methyl 5-(4-bromophenyl)-1-(4-chlorophenyl)-1H-pyrazole-3-carboxylic acid; 6h.** Pale pink crystals, yield 88% m.p. 378.96–98 °C. IR (KBr, v cm⁻¹): 1729.15(C=O); 1600.70(C=N); 1347.35(NH). 1H-NMR (400 MHz, DMSO-d₆ ppm): 3.96 (2H, CH₂ of OCH₃); 7.20 (2H, 1H, CH of C4 of pyrazole); 7.33 (4H, 2H, J= 8.6 Hz, C3, C5 of chlorophenyl); 7.53–7.56 (m, 4H, C2, C6 of chlorophenyl, C3 of bromophenyl); 7.74–7.82 (d, 2H, J= 8.36 Hz, C2, C6 of bromophenyl); EIMS, m/z (relative abundance %): 394 (M⁺+2) (28.07); 393 (M⁺+1) (11.87); 392 (M⁺) (70.52); 391 (9.85); 129 (21.55); 127 (91.87); 126 (17.15); 75 (37.57); 74 (34.46); 73 (26.25); 55 (52.76); 57 (59.94); 43 (100); 42 (32.85); 41 (30.96); Analysis Calcd for C₁₆H₁₂BrClN₄O: 272.73C, 352.14H, 3.09N, 7.15. Found: C, 51.98; H, 3.34; N, 7.28.

**General procedure for synthesis of 5-(4-Substitutedphenyl)-1-(4-substitutedphenyl)-1H-pyrazole-3-carboxyhydrazides (7a–i).** To methyl pyrazole-3-carboxylate 6a-i (1 mol) in ethanol (20–25 ml), hydrazine hydrate (5 mol) was added and the reaction mixture was refluxed for 7–9 h, then the solvent was evaporated under reduced pressure, then the precipitate was washed with cold water, then with ether, air-dried and recrystallised from ethanol.

**5-(4-Bromophenyl)-1-(4-chlorophenyl)-1H-pyrazole-3-carboxyhydrazide; 7h.** White crystals, yield 74%, m.p. 378.96–98 °C. IR (KBr, v cm⁻¹): 3246.82(NH); 1600.70(C=N); 1347.35(NH). 1H-NMR (400 MHz, DMSO-d₆ ppm): 4.49 (2H, 2H, NH₂, D₂O exchangeable); 7.01 (s, 1H, CH of C4 of pyrazole); 7.26–7.28 (m, 2H, C3, C5 of chlorophenyl); 7.35 (d, 2H, J= 8.76 Hz, C3, C5 of bromophenyl); 7.38–7.40 (m, 2H, C2, C6 of chlorophenyl); 7.52 (d, 2H, J= 8.76 Hz, C2, C6 of bromophenyl); 9.61 (s, 1H, NH₂, D₂O exchangeable); EIMS, m/z (relative abundance %): 394 (M⁺+2) (19.90); 393 (M⁺+1) (78.76); 392 (M⁺) (16.23); 391 (60.63); 363 (27.69); 362 (13.58); 361 (10); 360 (14.11); 359 (41.32); 75 (66.24); 111 (100.79); Analysis Calcd for C₁₆H₁₂BrClN₄O: 397.65C, 49.07H, 3.09N, 7.15. Found: C, 49.38H, 3.23N; N, 14.50.

**General procedure for synthesis of 5-(4-Substitutedphenyl)-1-(4-substitutedphenyl)-1H-pyrazole-3-carboxyhydrazides (8a–i).** Equimolar amount of 2-formylthymol and 3 and appropriate hydrazide 7a–i in absolute ethanol and few drops of glacial acetic acid were refluxed for 4–6 h, cooled to RT, the precipitate was filtered, washed with diethyl ether, and recrystallised from ethanol.

**4-(3-(2-Hydroxy-3-isopropyl-6-methylbenzylidene)-5-phenyl-1H-pyrazol-1-yl)benzenesulphonamide; 8c.** Pale white crystals, yield 77%, m.p. 245–247 °C. IR (KBr, v cm⁻¹): 3576.41 (OH); 3282.66(NH); 1600.70(C=O); 1597.06(C=N); 1H-NMR (400 MHz, DMSO-d₆ ppm): 1.12 (d, 6H, J= 6.76 Hz, 2CH₃ of isopropyl); 2.37 (3H, CH₃ of 6-methylthymol); 3.1–3.2 (m, 1H, CH of isopropyl); 6.71 (1H, J= 7.68 Hz, C3 of thymol); 7.14 (d, 1H, J= 7.68 Hz, C4 of thymol); 7.24 (1H, CH of C4 of pyrazole); 7.35–7.36 (m, 2H, C2, C6 of phenyl); 7.42–7.43 (m, 3H, C3, C4, C5 of phenyl); 7.52 (2H, NH₂ of sulfamoyl, D₂O exchangeable); 7.61 (d, 2H, J= 8.36 Hz, C3, C5 of sulfamoylphenyl); 7.61 (d, 2H, J= 8.36 Hz, C2, C6 of sulfamoylphenyl); 9.07 (s, 1H, CH of CH=CH=CH=O); 12.25 (s, 1H, OH, D₂O exchangeable); 12.58 (s, 1H, NH of NH=NH=NH); 1H-NMR (100 MHz, DMSO-d₆ ppm): 19.13 (CH₃ of 6-methylthymol); 22.77 (2CH₃ of isopropyl); 26.50 (CH of isopropyl); 109.42 (C4 of pyrazole); 115.69 (C5 of thymol); 121.70 (C5 of pyrazole); 125.45 (C2, C7, C of sulfamoylphenyl); 127.21 (C2, C3, C5 of sulfamoylphenyl); 128.46 (C4 of thymol); 129.22 (C3, C5 of phenyl); 129.26 (C4 of phenyl); 129.32 (C2, C6 of phenyl); 129.62 (C1 of phenyl); 133.75 (C3 of thymol); 136.16 (C6 of thymol); 141.97 (C4 of sulfamoylphenyl); 144.35 (C3 of pyrazole); 145.40 (C5 of sulfamoylphenyl); 146.54 (C5 of pyrazole); 149.68 (CH of C=CH=O); 156.49 (C2 of thymol); 157.43 (C=O); EIMS, m/z (relative abundance %): 518 (M⁺+1) (11.45); 517 (M⁺) (36.90); 326 (88.11); 160 (100.00); 77 (65.57); Analysis Calcd for C₁₉H₁₄N₂O₆S (517.60): C, 62.65; H, 5.26; N, 13.53; S, 6.19. Found: C, 62.84H; 5.38; N, 13.79; S, 6.27.
C2, C6 of sulfamoyl phenyl); 127.20(2C, C3, C5 of sulfamoylphenyl); 128.45(C4 of thymol); 130.67(2C, C2, C6 of p-methoxyphenyl); 133.75(C3 of thymol); 136.15(C6 of thymol); 142.11(C4 of sulfamoylphenyl); 144.23(C3 of pyrazole); 145.30(C1 of sulfamoylphenyl); 146.43(C5 of pyrazole); 146.92(CH of CH=N); 156.47(C2 of thymol); 157.49(C=O); 160.25(C4 of p-methoxyphenyl); EIMS, m/z (relative abundance %): 549 (M+1) (38.26); 548 (M+) (63.25); 521 (72.07); 404 (78.65); 241 (100.00); 135 (63.36); Analysis Calcd for C28H28N4O3 (468.56): C, 71.78; H, 6.02; N, 11.96. Found: C, 72.11; H, 6.24; N, 11.87.

Analysis Calcd for C28H28N4O3 (468.56): C, 71.78; H, 6.02; N, 11.96. Found: C, 72.11; H, 6.24; N, 11.87.

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Analysis Calcd for C28H28N4O3 (468.56): C, 71.78; H, 6.02; N, 11.96. Found: C, 72.11; H, 6.24; N, 11.87.
C3, C5 of chlorophenyl); 7.57(d, 2H, J = 8.60 Hz, C2, C6 of chlorophenyl); 9.07(s, 1H, CH of CH=N); 12.21(s, 1H, OH, D2O exchangeable); 12.60(s, 1H, NH of NH-N=, D2O exchangeable); 13C-NMR (100 MHz, DMSO-d6 ppm): 19.12(CH3 of 6-methylthymol); 22.77(2CH3 of isopropyl); 26.50(CH of isopropyl); 55.68(CH3 of OCH3); 108.45(C4 of pyrazole); 110.47(5(C of pyrazole); 114.69(2C, C3, C5 of p-methoxyphenyl); 115.72(C1 of thymol); 121.27(C5 of thymol); 121.44(C1 of p-methoxyphenyl); 128.07(2C, C2, C6 of chlorophenyl); 128.39(C4 of thymol); 129.73(2C, C2, C6 of p-methoxyphenyl); 130.58(2C, C3, C5 of chlorophenyl); 133.55(C4 of chlorophenyl); 133.73(C3 of thymol); 136.12(C6 of thymol); 138.62(C1 of chlorophenyl); 145.19(C3 of pyrazole); 146.11(C5 of pyrazole); 149.56(CH of CH=N); 156.47(C2 of thymol); 157.57(C=O); 160.13(C4 of p-methoxyphenyl); EIMS, m/z (relative abundance %): 503 (M+1) (40.05); 502 (M+) (100.00); 327 (77.20); 311 (37.85); Analysis Calcd for C28H27ClN4O3 (503.00): C, 66.86; H, 5.41; N, 11.14. Found: C, 67.14; H, 5.56; N, 11.41.

**Biological screening**

**In vitro COX-1 and COX-2 inhibitory assay**

Compounds 4a–c and 8a–i were screened for their ability to inhibit COX-1 and COX-2 enzymes in vitro. This was carried out using Cayman colorimetric COX (ovine) inhibitor screening assay kit (Catalog No. 560131) supplied by Cayman chemicals, Ann Arbor, MI according to reported method46 (Page: S20, Supplementary file).

**In vitro 5-LOX inhibitory assay**

The newly synthesised compounds were screened for their ability to inhibit 5-LOX enzymes. This was carried out using Abnova 5-LOX inhibitor screening assay kit (Catalog No. 760700) according to reported method47 (Page: S21, Supplementary file).

**In vivo anti-inflammatory activity**

**Formalin-induced paw oedema.** Compounds that showed in vitro selectivity indices higher or nearly equivalent to reference drugs towards COX 2 enzyme, were further evaluated for their in vivo anti-inflammatory activity applying the formalin-induced paw oedema screening protocol as an acute inflammation model using celecoxib and Diclofenac sodium as reference drugs according to reported procedures48,49 (Approved by AlexU-IACUC)50 (Page: S22–23, Supplementary file).

**Gastric ulcerogenic activity.** Compounds were evaluated for acute gastric ulcerogenic effect in adult female Wistar rats. Gross examination was performed for any evidence of hyperaemia, haemorrhage, definite haemorrhagic erosion, or ulcer according to reported procedures51 (Approved by AlexU-IACUC)50 (Page: S24, Supplementary file).

**Molecular modelling.** The molecular modelling studies were performed using the Molecular Operating Environment (MOE 2016.08) software (Chemical Computing Group, Montreal, Canada).52 and the crystal structures of the proteins were downloaded from the Protein Data Bank (PDB) website (Page: S24, Supplementary file).

**Results and discussion**

**Chemistry**

Scheme 1 illustrated the synthesis of the target thymol–1,5-disubstitutedpyrazole hybrids. The dioxobutanoate derivatives 53 1a–c were cyclised using reaction conditions used in Knorr pyrazole synthesis54 and celecoxib synthesis55 by reaction with either hydrazine hydrate or substituted phenylhydrazine hydrochloride in glacial acetic acid. Hydrazine hydrate resulted in both cyclisation and formation of the hydrazide derivatives 2a–c. On the other hand, substituted phenylhydrazine hydrochlorides produced only the cyclised methyl esters 6a–i which were further reacted with hydrazine hydrate to produce the corresponding hydrazides 7a–i.

The hydrazides 2a–c and 7a–i were condensed with 2-formylthymol38–41 to yield the target thymol-1,5-disubstitutedpyrazole hybrids 4a–c and 8a–i, respectively. IR spectra of compounds 4a–c showed absence of bands assigned to CHO and NH2 and

![Scheme 1](image-url)
presence of absorption bands assigned to \( C=\text{N} \), as well as to OH. \(^1\)H-NMR spectra of compounds 4a–c were characterised by the absence of signals assigned to NH\(_2\) protons at their previously recorded positions, and presence of 2D\(_2\)O exchangeable singlets of (NH) functional groups one for (CO-NH-N) and the other for pyrazole at (12.61–12.64) ppm, (13.70–13.93) ppm, respectively. \(^13\)C-NMR spectrum of compounds 4a–c showed the presence of signals of \( C=\text{O} \) and (C3, C4, C5 of pyrazole) at expected chemical shifts. In addition, MS for 4c showed the molecular ion peak (M\(^+\)) at m/z 392. On the other hand, IR spectra of compounds 4a–c were characterised by the absence of absorption bands assigned to (NH\(_2\) of pyrazole) and (CHO) group and presence of absorption bands assigned to (NH), \( C=\text{N} \) and (C=O) groups as well as (OH) of thymol at their expected absorption regions. \(^1\)H-NMR spectra of compounds 8a–i were characterised by the absence of signals assigned to NH\(_2\) protons at their previously recorded positions, and presence of (CH) proton of C4 of pyrazole at (7.15–7.24) ppm as singlet signal, as well as D\(_2\)O exchangeable singlet of NH proton at 12.57–12.60 ppm. \(^13\)C-NMR spectra of compounds 8a–i showed the presence of signals of (C3, C4, C5 of pyrazoles) and (C=\text{N} of thiazolidine) at expected chemical shifts. Besides, MS of compounds 8a–i showed the molecular ion peak (M\(^+\)) for 8a at m/z 517, at 597 for 8b, at 548 for 8c, at 438 for 8d, at 517 for 8e, at 468 for 8f, at 551 for 8h, and at 502 for 8i (Scheme 1).

### Biological screening

**In vitro COX-1 and COX-2 inhibitory assay**

Compounds 4a–c and 8a–i were screened for their \textit{in vitro} inhibitory activity against COX-1 and COX-2 enzymes using Cayman colorimetric COX (ovine) inhibitor screening assay kit (Catalog No. S60131) supplied by Cayman chemicals, Ann Arbor, MI. The half-maximal inhibitor concentrations (IC\textsubscript{50} \( \mu \text{M} \)) were determined and the selectivity index (SI) values were calculated as SO\textsubscript{50} (COX-1)/IC\textsubscript{50} (COX-2) and recorded in Table 1. All compounds showed high activity, in nanomolar range, against COX-2 and high SI to COX-2. Compounds 8b, 8g, 8c, and 4a displayed nearly equal activity to celecoxib with high SI (316, 268, 204, and 151, respectively) (Table 1).

**In vitro 5-LOX inhibitory assay**

Compounds 4a–c and 8a–i were screened for their \textit{in vitro} ability to inhibit 5-LOX enzymes. This was carried out using Abnova 5-LOX inhibitor screening assay kit (Catalog No. 760700)\(^{47}\). All compounds showed potent 5-LOX inhibitory activities higher than the reference quercetin. Consequently, compounds 8b, 8c, 8g, and 4a showed dual inhibitory activities against COX-2 and 5-LOX higher than the references celecoxib and quercetin (Table 1).

**In vivo anti-inflammatory activity**

**Formalin-induced paw oedema test (acute inflammation model)**

Compounds 4a–c and 8a–i were tested for their \textit{in vivo} anti-inflammatory activity using formalin-induced paw oedema test (Acute inflammation model) (Table 2, Figures 3–5). All compounds showed higher % inhibition than celecoxib except 8b which revealed nearly equal activity to celecoxib. Compounds 4a and 8i exhibited double % inhibition exhibited by Celecoxib. All compounds showed higher % inhibition than that of Diclofenac sodium except 8a, 8b, 8f, and 8h which elicited slightly less % inhibition than that of Diclofenac sodium. Compound 4a was the most potent with % inhibition 81.93% comparing with 36.37% and 52.37% of celecoxib and diclofenac sodium, respectively.

**In vivo gastric ulcerogenic activity**

Compounds 4a–c and 8a–i were evaluated for their ulcerogenic potential in rats. Gross examination revealed that compounds 4a, 4b, 8b, and 8g showed superior gastrointestinal safety profile (no ulceration) as the references celecoxib and diclofenac sodium in the population of fasted rats. On the other hand, compounds 4c, 8a, 8c–f, 8h, and 8i showed variable degrees of hyperaemia (Figure 6).

**Structure–activity relationship**

The \textit{in vitro} inhibition of COX-1/2 and 5-LOX assays and the \textit{in vivo} anti-inflammatory testing showed that, substitution at para position of phenyl at position 5 of pyrazole by either electron donating group (OCH\(_3\)) or electron withdrawing group (Br) or unsubstituted did not affect the activity. This could be due to the presence of the phenyl ring out of coplanarity with the pyrazole moiety so could not affect the electronic configuration of the whole molecule hence binding affinity and activity. On the other hand, substitution at N1 of pyrazole affected the activity. The unsubstituted derivative 4a showed \textit{in vitro} COX-2/5-LOX inhibitory activity and \textit{in vivo} potent anti-inflammatory activity. Furthermore, the activities of N1 aryl substituted derivatives were affected with the type of substitution at para position of N1 phenyl moiety. The substitution with either sulphonamide group or chloro showed higher \textit{in vitro} COX-2/5-LOX inhibitory activity and \textit{in vivo} anti-inflammatory activity than unsubstituted derivatives. This could be due to the importance of sulphonamide and chloro groups in polar and hydrophobic interactions with the active sites of the target COX-2/5-LOX enzymes.

**Molecular modelling**

MOE 2016.08 software\(^{52}\) was used for performing molecular modelling studies for the most active compounds 4a, 8b, and 8g. These compounds were docked into the active site of COX-2 (PDB entry 3LN1) and 5-LOX (PDB entry 3V99). The results were illustrated in Tables 3 and 4 and Figures 7–13.

### Table 1. \textit{In vitro} COX-1, COX-2, and 5-LOX enzyme inhibitory activities, IC\textsubscript{50} values, and selectivity indices (SI) of the tested compounds.

| Compound | COX-1 IC\textsubscript{50} (\( \mu \text{M} \)) | COX-2 IC\textsubscript{50} (\( \mu \text{M} \)) | Selectivity index | 5-LOX IC\textsubscript{50} (\( \mu \text{M} \)) |
|----------|------------------|------------------|------------------|------------------|
| Celecoxib | 14.7 ± 0.07 | 0.045 ± 0.007 | 327 | – |
| Diclofenac sodium | 3.9 ± 0.04 | 0.8 ± 0.009 | 4.87 | – |
| Quercetin | – | – | – | 3.34 ± 0.05 |
| 4a | 10.3 ± 0.1 | 0.068 ± 0.01 | 151.5 | 3.05 ± 0.067 |
| 4b | 11.81 ± 0.15 | 0.17 ± 0.01 | 69.5 | 1.84 ± 0.06 |
| 4c | 10.22 ± 0.046 | 0.19 ± 0.007 | 53.8 | 2.91 ± 0.068 |
| 8a | 11.21 ± 0.15 | 0.12 ± 0.007 | 93.4 | 1.96 ± 0.036 |
| 8b | 13.61 ± 0.096 | 0.043 ± 0.001 | 316.5 | 1.58 ± 0.026 |
| 8c | 12.87 ± 0.097 | 0.063 ± 0.001 | 204 | 1.91 ± 0.053 |
| 8d | 10.19 ± 0.036 | 0.11 ± 0.007 | 92.6 | 3.11 ± 0.046 |
| 8e | 11.24 ± 0.075 | 0.12 ± 0.007 | 93.6 | 2.56 ± 0.025 |
| 8f | 11.18 ± 0.57 | 0.14 ± 0.01 | 79.8 | 2.91 ± 0.064 |
| 8g | 12.1 ± 0.16 | 0.045 ± 0.01 | 268.8 | 1.60 ± 0.042 |
| 8h | 12.61 ± 1.36 | 0.16 ± 0.01 | 78.8 | 1.97 ± 0.026 |
| 8i | 12.58 ± 0.46 | 0.17 ± 0.012 | 74 | 2.76 ± 0.016 |

\(^{a}\)IC\textsubscript{50} is the concentration (\( \mu \text{M} \)) needed to cause 50% inhibition of COX-1 and COX-2 enzymatic activity. All values are expressed as a mean of three replicates with standard deviation less than 10% of the mean.

\(^{b}\)Selectivity Index (SI) = (COX-1 IC\textsubscript{50}/COX-2 IC\textsubscript{50}).
| Code | Volume of oedema (ml) | Abs diff. | % Inhibition of oedema volume | % Relative potency (4h) celecoxib | % Relative potency (4h) diclofenac |
|------|----------------------|-----------|-------------------------------|---------------------------------|---------------------------------|
| Vehicle DMSO (control) | | | | | |
| 1 | 4.25 | 4.67 | 2.22 | | |
| 2 | 4.20 | 6.37 | 2.17 | | |
| 3 | 4.18 | 6.31 | 2.13 | | |
| Mean ± SD | 4.21 ± 0.04 | 6.38 ± 0.08 | 2.17 ± 0.05 | | |
| Reference standard (celecoxib) | | | | | |
| 1 | 4.25 | 5.69 | 1.44 | 35.10 | |
| 2 | 4.34 | 5.67 | 0.99 | 54.40 | |
| 3 | 4.25 | 5.19 | 0.94 | 55.90 | |
| Mean ± SD | 4.30 ± 0.05 | 5.69 ± 0.02 | 1.38 ± 0.06 | 36.33 ± 0.02 | |
| Reference standard (diclofenac) | | | | | |
| 1 | 4.20 | 5.37 | 1.17 | 47.30 | |
| 2 | 4.34 | 5.33 | 0.99 | 54.40 | |
| 3 | 4.25 | 5.19 | 0.94 | 55.90 | |
| Mean ± SD | 4.26 ± 0.07 | 5.30 ab ± 0.09 | 1.03 ab ± 0.12 | 52.53 ab ± 0.49 | 144.6 ± 12.56 |
| 4a | 1 | 5.15 | 5.50 | 0.35 | 84.20 | |
| 2 | 5.10 | 5.65 | 0.55 | 74.70 | |
| 3 | 5.25 | 5.55 | 0.30 | 85.90 | |
| Mean ± SD | 5.17abc ± 0.08 | 5.57abc ± 0.08 | 0.40abc ± 0.13 | 81.60abc ± 0.64 | 225.5c ± 28.34 | 156.4 ± 20.52 |
| 4b | 1 | 5.20 | 6.18 | 0.98 | 55.90 | |
| 2 | 5.15 | 6.17 | 1.02 | 53.00 | |
| 3 | 5.15 | 6.15 | 1.00 | 53.10 | |
| Mean ± SD | 5.17abc ± 0.03 | 6.17 abc ± 0.02 | 1.00abc ± 0.02 | 54.01 abc ± 0.16 | 148.9 ± 11.16 | 103.5 ± 12.68 |
| 4c | 1 | 5.35 | 6.10 | 0.75 | 66.20 | |
| 2 | 5.30 | 6.20 | 0.90 | 58.50 | |
| 3 | 5.25 | 6.30 | 0.95 | 55.40 | |
| Mean ± SD | 5.30abc ± 0.05 | 6.17abc ± 0.06 | 0.87abc ± 0.10 | 60.03abc ± 0.56 | 165.7 ± 20.01 | 115.6 ± 21.54 |
| 8a | 1 | 5.02 | 6.35 | 1.33 | 40.10 | |
| 2 | 4.96 | 6.35 | 1.39 | 35.90 | |
| 3 | 5.02 | 6.30 | 1.28 | 39.90 | |
| Mean ± SD | 5.0abc ± 0.03 | 6.33 abc ± 0.03 | 1.33 abc ± 0.06 | 38.63 abc ± 0.23 | 106.8 ± 12.02 | 74.10 ± 14.20 |
| 8b | 1 | 5.02 | 6.35 | 1.33 | 40.10 | |
| 2 | 4.97 | 6.40 | 1.02 | 53.00 | |
| 3 | 5.02 | 6.45 | 1.00 | 53.10 | |
| Mean ± SD | 5.0abc ± 0.03 | 6.40 bc ± 0.05 | 1.40 ab ± 0.02 | 35.53 ab ± 0.14 | 98.10 c ± 14.22 | 68.50 ± 14.20 |
| 8c | 1 | 5.35 | 6.25 | 0.90 | 58.10 | |
| 2 | 5.25 | 6.35 | 1.10 | 50.70 | |
| 3 | 5.40 | 6.20 | 1.00 | 53.10 | |
| Mean ± SD | 5.33abc ± 0.08 | 6.33abc ± 0.08 | 1.00ab ± 0.07 | 53.97abc ± 0.37 | 149.0 ± 17.26 | 103.7 ± 16.65 |
| 8d | 1 | 5.35 | 6.25 | 0.90 | 59.50 | |
| 2 | 4.96 | 6.03 | 1.07 | 50.70 | |
| 3 | 5.02 | 6.02 | 1.00 | 53.10 | |
| Mean ± SD | 5.0abc ± 0.03 | 6.04 abc ± 0.04 | 1.40 ab ± 0.06 | 35.53 abc ± 0.37 | 98.10 d ± 14.22 | 68.50 ± 14.20 |
| 8e | 1 | 5.30 | 6.25 | 0.93 | 58.10 | |
| 2 | 5.25 | 6.35 | 1.10 | 49.30 | |
| 3 | 5.40 | 6.20 | 1.00 | 53.10 | |
| Mean ± SD | 5.33abc ± 0.08 | 6.33abc ± 0.08 | 1.00 ab ± 0.07 | 53.97abc ± 0.51 | 149.1 ± 20.95 | 103.8 ± 19.09 |
| 8f | 1 | 5.15 | 6.25 | 1.10 | 50.50 | |
| 2 | 5.15 | 6.35 | 1.20 | 44.70 | |
| 3 | 5.15 | 6.40 | 1.20 | 43.70 | |
| Mean ± SD | 5.17abc ± 0.03 | 6.17abc ± 0.04 | 1.17 ab ± 0.06 | 46.30 ± 3.67 | 124.0 ± 24.00 | 78.20 ± 14.20 |
| 8g | 1 | 5.10 | 6.15 | 1.05 | 52.70 | |
| 2 | 5.15 | 6.10 | 0.95 | 56.20 | |
| 3 | 5.25 | 6.25 | 1.00 | 53.10 | |
| Mean ± SD | 5.17abc ± 0.08 | 6.17abc ± 0.08 | 1.00 ab ± 0.05 | 54.01 ab ± 0.19 | 148.6 ± 2.99 | 103.3 ± 8.20 |
| 8h | 1 | 5.02 | 6.05 | 1.03 | 53.60 | |
| 2 | 4.96 | 6.15 | 1.19 | 45.20 | |
| 3 | 5.02 | 6.0 | 0.98 | 54.00 | |
| Mean ± SD | 5.0abc ± 0.03 | 6.07 abc ± 0.08 | 1.17 ab ± 0.11 | 50.93 ± 0.47 | 140.9 ± 20.93 | 97.67 ± 15.13 |
| 8i | 1 | 5.30 | 6.02 | 0.72 | 67.60 | |
| 2 | 5.30 | 5.93 | 0.63 | 71.00 | |
| 3 | 5.40 | 5.95 | 0.55 | 74.20 | |
| Mean ± SD | 5.33abc ± 0.06 | 5.97abc ± 0.05 | 0.63abc ± 0.09 | 70.93abc ± 0.33 | 195.4 ± 13.97 | 135.4 ± 6.60 |

*aStatistically significant difference in comparison with control group.
*bStatistically significant difference in comparison with celecoxib reference group.
*cStatistically significant difference in comparison with diclofenac reference group.
Docking to COX-2 active site. Compounds 4a, 8b, and 8g which showed dual in vitro COX-2/5-LOX inhibiting activity and the reference celecoxib were docked into COX-2 active site (pdb entry 3LN1) using MOE version 2016.0802 software. The docking solutions of the compounds 4a, 8b, 8g, and celecoxib (Table 3 and Figures 7–10) confirmed the potential activities against COX-2 as the mode of interactions of the best poses were comparable to the reference celecoxib. All the target compounds interacted with hydrogen bond with the key amino acid Leu338. In addition, they showed potential polar interaction with the key amino acids Arg499 and Ser339 in the polar side pocket of COX-2 active site. Interaction with this small pocket’s amino acids is essential for the selective inhibition of the enzyme. Moreover, overlay of compounds 4a, 8b, 8g, and celecoxib inside the active site of COX-2
Table 3. Docking results of the active compounds in COX-2 active site.

| Code    | Score in Kcal/mol | Hydrogen bonds interaction amino acids | Hydrophobic interaction amino acids | Polar interaction amino acids |
|---------|------------------|---------------------------------------|------------------------------------|-------------------------------|
|         |                  | Four bonds: two bonds between N of SO₂ NH₂ and Ser339, Leu338; each O of SO₂ with Arg499 and Ile503 | Val335, Leu370, Phe367, Leu517, Phe345, Trp373, Val102, Met508, Ala502, Phe504, Val509 | His75, Tyr341, Ser516, Tyr371, Gly340, Arg106, Gln178, Gly512, arene-H between phenyl of methylphenyl and Ala513 |
|         |                  | One bond: between NH of CONH and Leu338 | Pro177, Val335, Ala513, Ala513, Phe504, Ala502, Pro500, Val509 | Asp501, Thr79, His75, Arg499, Gln178, Gly340, Tyr341, arene-H between pyrazole and Ser339 |
| 8b      |                  | One bond: between NH of CONH and Leu338 | Val335, Leu517, Trp373, Met508, Ala502, Pro500, Leu345, Val102 | Gln512, Asp501, Thr79, His80, Arg499, Tyr341, His75, Gln178, Gly340, Ser516, Arg106, Tyr371, arene-H between pyrazole and Ser339 |

*Amino acids interacted with the co-crystallised ligand (celecoxib) were marked in bold format.

Table 4. Docking results of the active compounds in 5-LOX active site.

| Code    | Score in Kcal/mol | Hydrogen bonds interaction amino acids | Hydrophobic interaction amino acids | Polar interaction amino acids |
|---------|------------------|---------------------------------------|------------------------------------|-------------------------------|
|         |                  | No hydrogen bonds                     | Ile406, Phe177, Val175, Leu607. | Gln557, Gly174, Asn554, His367, Asp176, Gln363, Lys173, Ser171, Glu172 |
| 4a      |                  | One hydrogen bond between N1 of pyrazole and *Asp176 | Ala410, Phe555, Ala672, Phe177, Phe610, Leu607, Ile406 | Asn554, His372, Gly174, Asn367, Gln557, Lys173, Asp176, Gly363, Glu364, Lys173, Glu413 |
| 8b      |                  | One hydrogen bond between N of sulphonamide and Ala672 | Val604, Leu368, Phe177, Val671, Ala672, Phe610, Leu607, Phe555, Phe555, Pro668 | Tyr558, Gln557, Ser608, His367, His550, Gln363, Glu364, Gly174, Asn554, Lys173, FE701, Thr364 |
| 8g      |                  | Coordinate bond between carbonyl oxygen and FE701. | Leu607, Val604, Phe555, Phe610, Val671, Ala140, Leu368. | Glu614, Gly174, Gln363, Tyr558, His367, Gln557, Asn554, His550, His372 |

*Amino acids interacted with the co-crystallised ligand (Arachidonic acid) were marked in bold format.
(Figure 9) revealed that, the pyrazole moiety of 4a and 1-arylpyrazole moiety of 8b and 8g were superposed on 1-arylpyrazole moiety of celecoxib with p-substituents interacted in the same position of trifluoromethyl of celecoxib. Besides, the carbonyl group interacted in the same position of sulphonamide group of celecoxib.

**Docking to 5-LOX active site.** Compounds 4a, 8b, and 8g which showed dual in vitro COX-2/5-LOX inhibiting activity and the co-crystallised ligand arachidonic acid were docked into 5-LOX active site (pdb entry 3V99)\(^{39}\) using MOE version 2016.0802 software\(^{32}\). The docking solutions of the compounds 4a, 8b, 8g, and arachidonic acid (Table 4 and Figures 11–13) supported the potential activities against 5-LOX. It was reported that, 5-LOX active site comprises several anchors. Polar positively-charged anchor consists of His550, His367 and His372 which interact with Fe\(^{2+}\). Polar anchor form both electrostatic and hydrogen-bonding interactions comprises Asp176, Asn180 and Gln363. Hydrophobic anchor contains Phe177, Leu607, Ile673, Leu414, Phe421, Gly174, Val175, Leu368, and Ile406\(^{59}\).

Compounds 4a, 8b, and 8g interacted with various anchors in 5-LOX...
Figure 10. Mode of binding (3D) of celecoxib (A), 4a (B), 8b (C), and 8g (D) into COX-2 active site.

Figure 11. Mode of binding (2D) of Arachidonic acid (A), 4a (B), 8b (C), and 8g (D) inside the active site of 5-LOX.
active site. All of them showed polar interaction with His367 beside additional interaction with His372 for 4a and His550 for 8b and 8g. Besides, all compounds elicited polar interaction with Gln363 in addition to hydrogen bond formed between N1-pyrazole of 4a and Asp176 as part of the second polar anchor. Furthermore, 4a, 8b, and 8g interacted by hydrophobic anchor with Phe177 and Leu607 in addition to hydrophobic interaction with Leu368 for 8b and 8g and Ile406 for 4a. It is worth mentioning that, arachidonic acid did not form hydrogen bond with 5-LOX active site, while the target compounds formed hydrogen bond and coordinate bond interactions with 5-LOX active site so the target compounds could have favourable binding affinity towards 5-LOX more than arachidonic acid.

**Conclusion**

Selective COX-2 inhibitors have many benefits in treatment of inflammation, but this selective inhibition resulted in accumulation of arachidonic acid at 5-LOX site which leads to overproduction of LTs which in turn induced asthmatic problems, gastric damage, and ulceration. New thymol – 1,5-disubstitutedpyrazole hybrids were synthesised as dual COX-2/5-LOX inhibitors to get safer anti-inflammatory therapy. Compounds 8b, 8g, 8c, and 4a displayed in vitro inhibitory activity against COX-2 nearly equal to celecoxib with high SI comparable to celecoxib. All compounds, 4a–c and 8a–i, showed 5-LOX inhibitory activity higher than reference quercetin. Furthermore, all compounds, 4a–c and 8a–i, showed in vivo inhibition of formalin induced paw oedema higher than celecoxib. Compound 4a was the most potent with inhibition percentage higher than celecoxib and diclofenac sodium. In addition, compounds 4a, 4b, 8b, and 8g showed superior gastrointestinal safety profile (no ulceration) as the references celecoxib and diclofenac sodium in the population of fasted rats. *In silico* docking studies in the COX-2 and 5-LOX active sites predicted that, the target compounds could have strong binding affinity to the target enzymes active sites in comparison with the reference celecoxib and arachidonic acid, respectively. In conclusion, compounds 4a, 8b, and 8g achieved the target goal. They elicited in vitro dual inhibition of
COX-2/5-LOX higher than celecoxib and quercetin, in vivo potent anti-inflammatory activity higher than celecoxib and in vivo superior gastrointestinal safety profile (no ulceration) as celecoxib.

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