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Design, synthesis and biological investigation of certain pyrazole-3-carboxylic acid derivatives as novel carriers for nitric oxide

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Dedicated to Professor Jochen Lehmann on the occasion of his 65th birthday

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ABSTRACT

Some novel pyrazole-NO hybrid molecules **5a–e**, **6**, **8** and **10** were prepared through binding of the pyrazole-3-carboxylic acid derivatives with nitric oxide donor moiety like oxime or nitrate ester. The prepared compounds were evaluated for nitric oxide release, antibacterial and anti-inflammatory activities. The organic nitrate **10** exhibited the highest percentage of NO release using Griess diazotization method. Some of the prepared compounds exhibited remarkable antibacterial activity against *Escherichia coli* C-600, *Pseudomonas aeruginosa*, *Bacillus subitilis* and *Staphylococcus aureus* NCTC 6571 compared to ciprofloxacin. Most of the tested compounds showed significant anti-inflammatory activity compared to indomethacine using carrageenan induced paw edema method. In general, structural modification of compound **2** either to nitrate ester or oxime hybrids showed better anti-inflammatory with less ulcerogenic liability than their corresponding starting intermediates.

1. Introduction

Pyrazole derivatives are well established in the literatures as important biologically effective heterocyclic compounds. These derivatives are the subject of many research studies due to their widespread potential pharmacological activities such as anti-inflammatory,[1] antipyretic,[2] antimicrobial,[3] antiviral,[4] antitumour,[5] anticonvulsant,[6] antihistaminic[7] and antidepressant[8] activities. The widely prescribed anti-inflammatory pyrazole derivatives, celecoxib[9] and deracoxib[10] are selective COX-2 inhibitors with reduced ulcerogenic side effects.

Nitric oxide (NO) is a magic molecule endogenously discovered as endothelium derived relaxing factor.[11] Besides its vasodilatation effect,[12] NO is involved in many physiological and pathophysiological processes[13] like inhibition of platelets aggregation[14,15] and immune defense against viruses,[16] bacteria[16] and cancerous cells.[17] Now, it is clear that the vasodilatory effects of the old known organic nitrate and nitrite esters, glyceryltrinitrate, isosorbid dinitrate and amyl nitrite are mediated through liberation of NO after metabolic bioactivation.[18]

Recent strategies devoted to minimize the ulcerogenic side effects of non-steroidal anti-inflammatory drugs (NSAIDs) by binding them to NO donating moieties.[19] In such hybrids, NO supports several endogenous GIT defense mechanisms against ulcer formation including increase in mucous and bicarbonate secretions, increase in mucosal blood flow and inhibition of pro-inflammatory caspase process.[20] A series of NO/aspirin (e.g., NCX-4016)[19] acquired anti-inflammatory effects devoid of acute gastrotoxicity and showed potent antiaggregatory effects, principally as a consequence of their NO donor ability.

Based on the above mentioned research results, the objective of this study aimed to synthesize some novel pyrazole-3-carboxylic amides and esters as a type of prodrugs in order to improve the expected ulcerogenic liability. In addition some novel pyrazole-NO hybrids molecules were prepared by binding the pyrazole-3-carboxylic derivatives with NO donor moiety like oxime or nitrate ester, for the purpose of synergism and/or decreasing the expected ulcerogenic side effects. Also, a histopathological investigation for ulcer formation liability was carried out. The synthesis of the target compounds was illustrated in Schemes 1 and 2.

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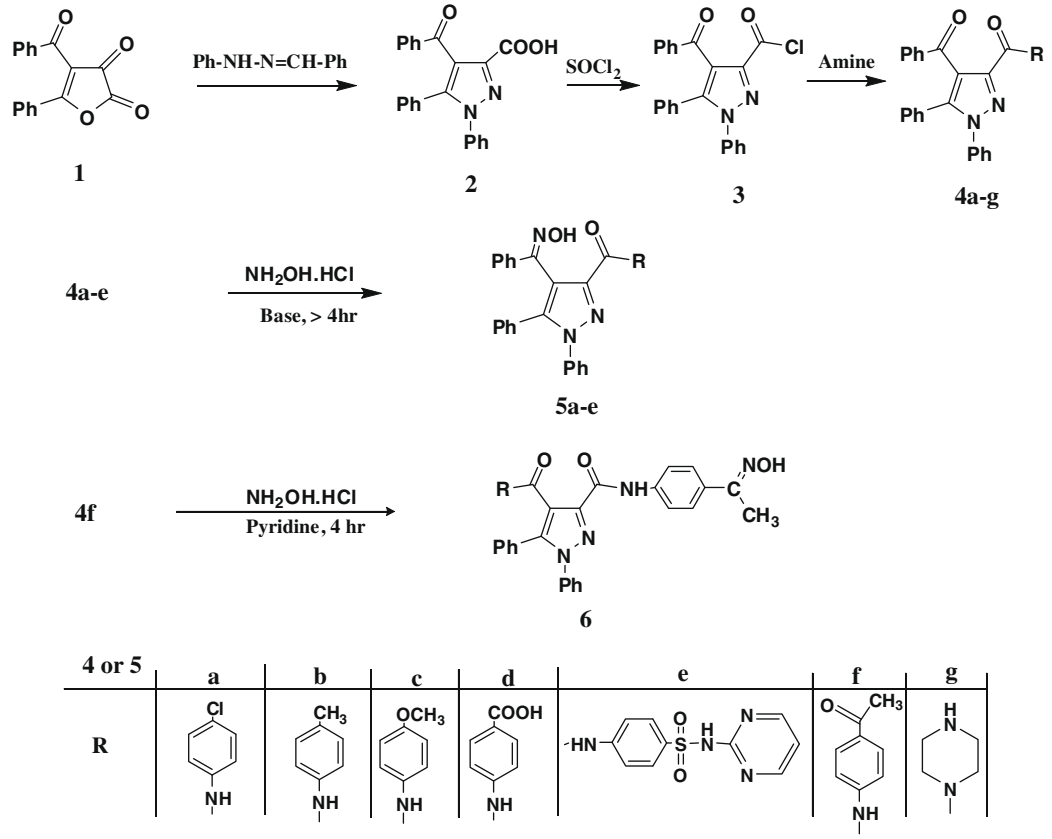
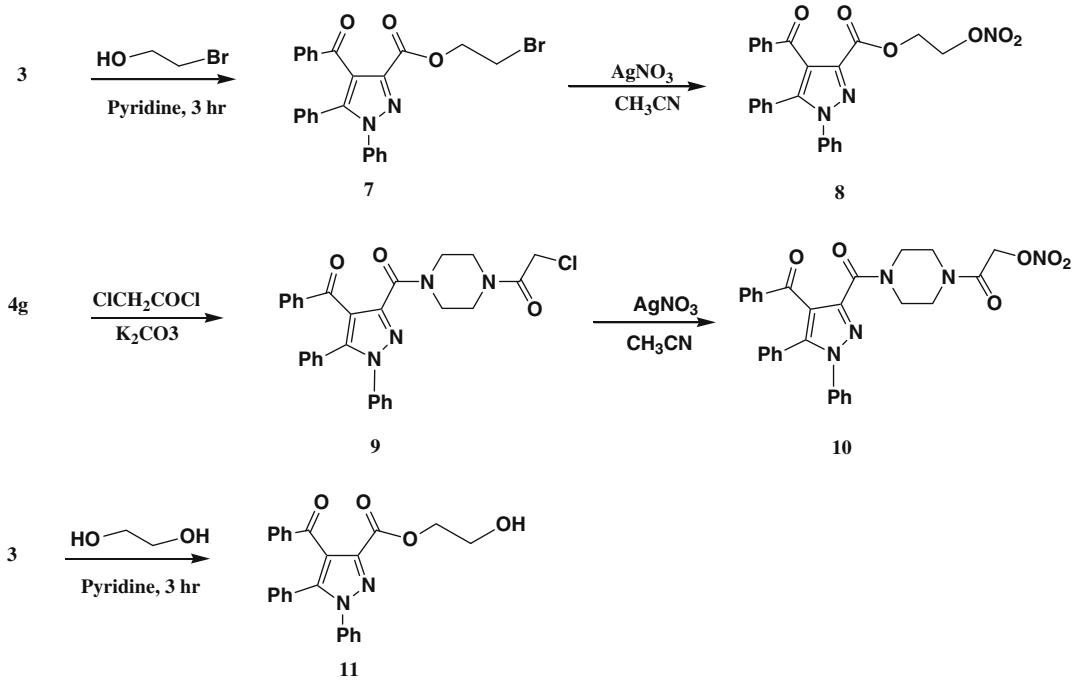


Scheme 1. Synthesis of the pyrazole-3-carboxylic acid derivatives **4a–g**, **5a–e**, and **6**.



Scheme 2. Synthesis of the pyrazole-3-carboxylic acid derivatives **7–10**.

2. Results and discussion

2.1. Chemistry

The key intermediate, pyrazole-3-carboxylic acid **2** was prepared as reported[21] by heating the freshly prepared 4-benzoyl-5-phenylfurane-2,3-dione[22] **1** with 1-benzylidine-2-phenylhydrazone without any solvent. Heating at reflux of the pyrazole-3-carboxylic acid **2** with thionyl chloride on a steam bath afforded the corresponding acid chloride **3**.[23] Reaction of the acid chloride **3** with the appropriate nucleophiles afforded the corresponding amides **4a–g** in 50–78% yield. Condensation of the amides **4a–e**

with hydroxylamine hydrochloride in absolute ethanol for (4–30 h) using triethylamine or pyridine as a base afforded the corresponding oximes **5a–e** in 60–82% yield. The oxime **6** was prepared by the same procedure with a shorter reaction time (less than 4 h).

The IR spectra of the prepared oximes **5a–e** and **6** revealed the disappearance of the respective carbonyl stretching absorption and appearance of the characteristic oximic OH stretching as broad medium band ranging from 2500 to 3200 cm^{-1}. The ^1H NMR spectra of the prepared oximes revealed the characteristic proton of –C═N–OH as a broad signal exchangeable with D_2O in the range of 8.5–12.3 ppm. It is noteworthy to focus on the change in the chemical shift of the methyl group of the oxime **6**, it is upfield shifted from 2.5 to 2.3 ppm due to the change of the adjacent C═O to the less electronegative –C═N–OH.

The mass spectrum of some of the oximes showed the disappearance of M^+ and appearance of $M^{+\cdot}-OH$ peak due to the rapid fragmentation of M^+ to the more stable fragment of $M^{+\cdot}-OH$. On the other hand, the oxime **5e** as a representative example revealed the molecular ion peak at $m/z = 615$ in addition to the $M^{+\cdot}-OH$ fragment at $m/z = 598$, that underwent loss of the amide moiety giving a fragment at $m/z = 348$. This last fragment will subsequently fragmented either by cleavage of the pyrazole ring affording a fragment at $m/z = 180$ or complete loss of the pyrazole ring giving a fragment for the remaining at $m/z = 103$. Subsequent loss of (C═N) from the last fragment gives a fragment at $m/z = 77$ (100%).

The titled ester **7** was prepared by heating at reflux of the acid chloride **3** with bromoethanol. Heating at reflux the ester **7** with $AgNO_3$ in acetonitril afforded the corresponding nitrate ester **8**. The ^1H NMR spectrum of the nitrate ester **8** showed the $-CH_2CH_2-$ pattern as a singlet at 4.4 ppm, where the two methylenes are considered magnetically equivalent.

Treatment of the amide **4g** with chloroacetyl chloride in K_2CO_3 at 0 °C gave the corresponding acetylated derivative **9** in low yield. The objective nitrate ester **10** was prepared by heating at reflux of the intermediate **9** with $AgNO_3$ in acetonitril. The downfield shift of the absorption of the $-CH_2$ group at 5.2 ppm was the basis for the identification of the nitrate ester **10**. The hydroxyethyl ester **11** of compound **2** was prepared for comparison with its corresponding nitrate ester **8** by heating at reflux of the acid chloride **3** with ethylene glycol.

2.2. Nitric oxide release

The percentage of nitric oxide release from the prepared NO donating hybrids **5a–e**, **6**, **8** and **10** was determined. The spontaneous oxidation of NO into nitrite was the basis for the colorimetric measurement using Griess diazotization reaction method.[24] The experiment was carried out in phosphate buffer of pH 7.4 in the presence of L-cysteine using sodium nitrite solution (1–50 nmol/mL) as a standard to establish the sodium nitrite standard curve according to GRAPH PAD INSTANT program. The formed intense purple colour was measured at λ_{max} 540 nm after 25 min. The percentage of NO release is calculated from the following equation:

Table 1
Percentage of NO release of compounds **5a–e**, **6**, **8** and **10**

Compound	Percentage of NO release
5a	7.80
5b	10.06
5c	8.00
5d	9.30
5e	7.40
6	11.58
8	7.10
10	13.84

Table 2
Minimal inhibitory concentration of the tested compounds **2**, **4a–g**, **5a–e** and **6–11**

Compound	Minimal inhibitory concentration (MIC) (μg/mL)			
	B. subitilis	*S. aureus*	*E. coli*	*P. aeruginosa*
Ciprofloxacin	0.61	52.40	1.20	30.80
2	10.76	−ve	16.10	1.92
4a	−ve	−ve	−ve	25.09
4b	33.80	−ve	16.14	4.30
4c	43.80	−ve	4.50	22.90
4d	34.80	64.00	30.90	25.33
4e	47.03	−ve	−ve	44.34
4f	1.75	−ve	39.90	32.40
4g	2.18	−ve	1.92	84.53
5a	5.82	90.36	14.09	10.32
5b	2.87	−ve	28.64	7.99
5c	43.86	−ve	2.88	9.70
5d	29.50	50.50	2.80	13.60
5e	54.67	−ve	30.86	23.34
6	90.36	−ve	1.62	27.22
7	47.50	−ve	24.04	6.04
8	21.68	−ve	15.28	11.31
9	11.19	−ve	1.37	61.52
10	2.45	−ve	63.97	93.33
11	3.88	−ve	7.95	1.29

−ve means no antibacterial activity.

$$\% \text{ of NO release} = \frac{\text{Found concentration of NO}}{\text{Theoretical concentration of NO}} \times 100$$

Results revealed that all of the tested compounds **5a–e**, **6**, **8** and **10** release NO with relatively similar percentage. The nitrate ester **10** exhibited the highest percentage of NO release (Table 1).

2.3. Screening of the antibacterial activity

The synthesized compounds **2**, **4a–g**, **5a–e**, **6–11** were screened for their antibacterial activity against *Escherichia coli* C-600, *Pseudomonas aeruginosa* (as examples for Gram-negative bacteria), *Bacillus subitilis* and *Staphylococcus aureus* NCTC 6571 (as examples for Gram-positive bacteria) using the agar cup diffusion method.[25] The MICs (μg/mL) of the tested compounds in comparison to ciprofloxacin are listed in Table 2.

Data in Table 2 revealed that the esters **7** and **11** exhibited the highest antibacterial activity against *P. aeruginosa* with MIC value of 6.04 and 1.29 μg/mL, respectively that is more active than ciprofloxacin, while the amide **4c**, **4g** the oximes **5c**, **5d**, **6** and **9** showed the highest activity with MIC values of 4.50, 1.92, 2.88, 2.80, 1.62, 1.37 μg/mL, respectively against *E. coli*. All of the tested compounds revealed no antibacterial activity against *S. aureus* except the amide **4d** and the oximes **5a** and **5d** which showed a markedly weak activity. Compound **4f**, **4g**, **5b** and **10** are the most active derivative against *B. subitilis* strains with MIC of 1.75, 2.18, 2.87, 2.45 μg/mL, respectively. Some of the nitric oxide donating hybrids exhibited better antibacterial activity than their corresponding intermediates that may be attributed to the synergistic antibacterial effect of NO.[16]

2.4. Screening of anti-inflammatory activity

The synthesized compounds **2**, **4a–g**, **5a–e**, **6–8**, **10** and **11** were evaluated for their in vivo anti-inflammatory activity using the carrageenan-induced paw edema method described by Winter et al.[26] Rat paw edema was induced by subcutaneous injection of 0.1 mL of 1% suspension of carrageenan in saline into the left paw. The right paw was injected with an equal volume of saline and served as a control. After 3 h, the average percentage increase in foot edema thickness measurement reaches maximum and served as a

Table 3
Percentage of Edema inhibition ± SEM after administration of indomethacin, 2, **4a–g**, **5a–e**, **6–8**, **10** and **11**

Compound	Dose (μmol/kg)	Percentage of edema inhibition				
		1 h	2 h	3 h	4 h	5 h
Indomethacin	27.94	40.00 ± 0.33**	36.60 ± 0.31**	63.17 ± 0.33**	66.17 ± 0.35**	69.86 ± 0.33**
2	27.17	32.72 ± 0.57**	35.00 ± 0.88**	36.50 ± 0.88**	51.47 ± 0.33**	56.62 ± 0.88**
4a	20.92	45.45 ± 0.33**	39.99 ± 0.33**	34.99 ± 0.58**	36.50 ± 1**	55.88 ± 0.33**
4b	22.29	42.54 ± 0.17**	29.09 ± 0.33**	28.33 ± 0.33**	30.16 ± 0.33**	51.17 ± 0.57**
4c	21.12	39.99 ± 0.44**	42.54 ± 0.67**	28.33 ± 0.88**	42.99 ± 1**	52.94 ± 0.33**
4d	20.52	23.63 ± 0.58*	25.00 ± 0.76*	26.98 ± 0.58*	41.18 ± 0.20*	46.38 ± 0.33*
4e	16.66	18.18 ± 0.29**	18.18 ± 0.33**	24.99 ± 0.33**	26.98 ± 0.58**	41.18 ± 0.88**
4f	20.60	29.09 ± 1**	45.45 ± 0.66**	44.99 ± 0.58**	47.62 ± 0.88**	61.76 ± 0.58**
4g	22.72	34.99 ± 0.72**	41.67 ± 0.88**	28.57 ± 0.88**	51.47 ± 0.88**	66.67 ± 0.66**
5a	20.28	23.63 ± 0.45**	23.33 ± 0.33**	47.61 ± 0.33**	55.88 ± 0.33**	60.87 ± 0.58**
5b	21.16	12.72 ± 0.67**	19.99 ± 0.29**	36.50 ± 0.58**	45.59 ± 0.67**	82.60 ± 0.33**
5c	20.47	30.90 ± 0.33**	25.00 ± 0.5**	36.50 ± 0.58**	51.47 ± 0.33**	56.52 ± 1**
5d	19.89	45.45 ± 0.29**	44.99 ± 0.33**	47.62 ± 0.29**	61.76 ± 0.33**	81.16 ± 0.67**
5e	16.24	16.36 ± 0.33**	26.67 ± 0.29**	31.74 ± 0.58**	51.47 ± 0.33**	56.52 ± 0.58**
6	19.98	16.36 ± 0.88**	33.30 ± 1.00**	33.30 ± 0.57**	51.47 ± 0.67**	62.32 ± 0.58**
7	21.04	25.45 ± 0.44**	28.38 ± 0.67**	30.16 ± 0.67**	41.18 ± 1.00*	52.17 ± 0.67**
8	21.86	29.09 ± 0.44**	34.99 ± 0.58**	47.62 ± 0.88**	58.82 ± 0.60**	71.01 ± 0.58**
10	18.53	45.45 ± 0.50**	29.99 ± 0.44**	42.99 ± 0.67**	45.59 ± 0.88**	68.12 ± 0.58**
11	24.24	19.66 ± 0.57**	51.51 ± 0.66**	66.43 ± 0.00**	68.60 ± 0.33**	71.74 ± 0.33**

Significantly different from control at $^{*}p < 0.05$, $^{**}p < 0.01$.

control. The treated groups received the appropriate dose (100 mg/kg) of the tested compounds orally half an hour prior to carrageenan injection. The measurement was carried out at 0, 1, 2, 3, 4 and 5 h after administration of the tested compounds, indomethacine and vehicle. The obtained results were subjected to statistical analysis by student's *t*-test using the 'ANOVA T program'. The difference in the results was considered significant when the values of *P* are less than 0.05 or *P* less than 0.01. The percentage of edema inhibition ± standard error of the mean was calculated[28] and listed in Table 3. The following equations is used for calculation of percentage edema inhibition.

$$\%\ \text{Edema inhibition} = \frac{(V_R - V_L)\ \text{control} - (V_R - V_L)\ \text{treated}}{(V_R - V_L)\ \text{control}} \times 100$$

where V_R is the average right paw thickness; V_L is the average left paw thickness.

Results revealed that all of the tested compounds showed a variant significant anti-inflammatory activity at the administered dose (100 mg/kg) compared to indomethacin as a reference. It is obvious that most of the tested amides **4a–g** revealed relatively higher activities than their starting, pyrazole-3-carboxylic acid **2**. The results also revealed that in some cases, the presence of the NO donating moiety enhances the anti-inflammatory activity in comparison with their corresponding starting derivatives like in compounds **5d** and **5e**. It is obvious that, there is no direct correlation between enhancement of the anti-inflammatory activity and the presence of NO donating moiety. Also, it is clear that maximal anti-inflammatory activity was obtained after 4–5 h of administration.

2.5. Assessment of gastric mucosal ulcerogenicity

In order to investigate the ulcerogenic liability of the tested compounds **2**, **4a–g**, **5a–e** and **6–10**, the rats used in testing the anti-inflammatory activity were sacrificed, their stomach were removed, opened and examined for the presence of visible lesions. Gastric mucosal lesions were expressed in terms of the ulcer index (UI) according to the method of Till et al.[27] which depends on the calculation of lesion index by using of a 0–5 scoring system based on the severity of each lesion. The severity factor was defined according to the length of the lesions. Severity factor is as follows, 0 = no lesions; 1 = petechiae; 2 = erosions < 1 mm; 3 = erosions of 1–2 mm; 4 = erosions of 2–4 mm and 5 = erosions > 4 mm. The partial scores were then summed to obtain the ulcer index for the examined animal. The UI for each group was taken as the mean lesion score for all rats in those groups. The preventive index (PI) of the tested compounds was calculated from the following equation according to Hano et al.[28]

$$\text{Preventive index(PI)} = \frac{\text{UI of ulcered group} - \text{UI of treated group}}{\text{UI of ulcered group}} \times 100$$

The results listed in Table 4 revealed that all of the tested compounds exhibited marked low UI in comparison to the reference (indomethacin) that indicates their gastroprotective effect. Unexpectedly, the pyrazole-3-carboxylic acid **2** exhibited lower UI. Most of the synthesized pyrazole-NO hybrid compounds containing

Table 4
Ulcer index of indomethacin, carboxymethylcellulose, **2**, **4a–g**, **5a–e**, **6–8**, **10** and **11**

Compound	UI
Indomethacin	69.5
Carboxymethylcellulose	2.6
Control	0.3
2	8.0
4a	20.3
4b	4.0
4c	33.3
4d	17.0
4e	9.3
4f	11.0
4g	23.0
5a	7.5
5b	3.0
5c	8.5
5d	8.5
5e	7.6
6	4.0
7	11.0
8	7.5
10	5.5
11	9.0

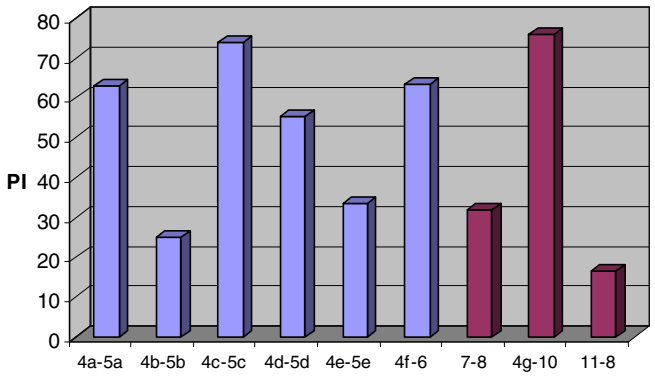


Figure 1. The preventive index of the tested NO donating hybrids calculated by comparison with their starting derivatives.

either oxime or nitrate moiety have higher percentage of prevention index. This may be attributed to the release of NO in vivo which possesses gastroprotective activity (Fig. 1).

2.6. Histopathological investigation

The histological slides were prepared according to the reported procedures.[29] The slides obtained from the control group showed no lesions and characterized by continuous mucosal layer (Fig. 2a). The slides of the intermediates **4a** (Fig. 2b) and **4g** (Fig. 2d) showed greater irregular mucosa with ulcers of approximately higher number and severity with UI values of 23 and 20.3, respectively. On the contrary, the slides of the oxime **5a** (Fig. 2c) and the nitrate ester **10** (Fig. 2e) exhibited ulcers of low number and severity with UI of 7.5 and 5.5, respectively. This may be attributed to the ability of NO donating hybrids to release NO with well known gastroprotective effect.

3. Conclusion

A group of pyrazole-3-carboxylic acid derivatives in addition to their corresponding NO hybrid molecules were prepared. Some of the prepared compounds exhibited good antibacterial activity compared to ciprofloxacin. Most of the synthesized compounds exhibited significant anti-inflammatory activity using indomethacin as a reference. The NO hybrid molecules either oxime or nitrate ester showed pronounced gastroprotective activity better than their corresponding starting intermediate amides or esters. This may be attributed to the release of NO, the oxime **5c** and the nitrate ester **10** showed the highest PI (74%, 76.08%, respectively). Histopathological examination of some amide intermediates and their corresponding oximes indicates that the NO donating moiety either oxime or nitrate reduced greatly the incidence of gastric ulceration.

4. Experimental

4.1. Chemistry

Melting points were recorded on Stuart electrothermal melting point apparatus and were uncorrected. IR spectra were recorded as KBr disks on a Brukar Vector 22 IR spectrometer and also on Perkin Elmer 298 spectrometer. ^{1}H NMR spectra were run on GEMINI-200 NMR spectrometer (200 MHz) and on MERCURY-300BB NMR spectrometer (300 MHz) and also on Varian Em-360L NMR spectrometer (60 MHz) using tetramethylsilane (TMS) as internal standard. Chemical shifts are expressed in δ ppm. Mass spectra were obtained on HP mass spectrometer which was recorded in the positive ion mode with leucine enkephalin as an internal lock mass standard. Elemental microanalyses were performed on a Perkin Elmer 2400 CHN Elemental analyzer. All chemicals and solvents were obtained commercially and were of the highest pure form. The reactions follow up and checking the purity of the compounds were made by TLC (Kieselgel 60 F254 precoated plates, E. Merck, Dermastadt, Germany), the spots were detected by exposure to UV lamp at λ_{max} 254 nm. Absorbance was measured by 'Spectronic Genesys' spectrophotometer connected to an IBM computer loaded with the Winspec application software (Miton Roy, USA). Bright field microscopy was used for investigation and capturing images for the histology and immunoperoxidase staining. Slides were photographed using Olympus digital camera. The furandione **1** was prepared as reported.[22] Also, the pyrazole-3-carboxylic acid[21] **2** and the corresponding acid chloride[23] **3** were prepared as reported.

4.1.1. General procedure for synthesis of 4-benzoyl-1,5-diphenylpyrazole-1*H*-3-carboxamide derivatives 4a–g

A mixture of the pyrazole-3-carboxylic acid chloride **3** (0.386 g, 1 mmol) and the respective amine (1 mmol) was heated at reflux in toluene for 4–5 h. The solvent was evaporated under reduced pressure and the obtained crude product was recrystallized from absolute ethanol.

4.1.1.1. *N*-(4-Chlorophenyl)-4-benzoyl-1,5-diphenyl-1*H*-3-pyrazolecarboxamide 4a. Compound **4a** was prepared according to the general procedure above in 73% yield; mp, 177–178 °C; IR (ν cm^{-1}): 3365 (NH), 1664 (C=O, benzoyl), 1655 (C=O, amide), and 1595 (C=N); ^{1}H NMR (200 MHz, $CDCl_3$): δ 9.00 (s, 1H, NH), 7.1–7.86 (m, 19H, Ar-H). FAB-MS: m/z 478 (M+1).

4.1.1.2. *N*-(4-Methylphenyl)-4-benzoyl-1,5-diphenyl-1*H*-3-pyrazolecarboxamide 4b. Compound **4b** was prepared according to the general procedure above in 50% yield; mp, 183–185 °C; IR (ν cm^{-1}): 3434 (NH), 1715 (C=O, benzoyl), 1635 (C=O, amide), and 1596 (C=N); ^{1}H NMR (60 MHz, $CDCl_3$): δ 9.5 (s, 1H, NH), 7.3–8.5 (m, 19H, Ar-H) and 2.4 (s, 3H, CH_3). Anal. Calcd for. $C_{30}H_{23}N_3O_2{\cdot}0.5H_2O$: C, 77.23; H, 5.19; N, 9.01. Found: C, 77.22; H, 5.19; N, 8.71.

4.1.1.3. *N*-(4-Methoxyphenyl)-4-benzoyl-1,5-diphenyl-1*H*-3-pyrazolecarboxamide 4c. Compound **4c** was prepared according to the general procedure above in 78% yield; mp, 179–181 °C; IR (ν cm^{-1}): 3381 (NH), 2986 (C–H str.), 1680 (C=O, benzoyl), 1669 (C=O, amide), and 1593 (C=N); ^{1}H NMR (200 MHz, $CDCl_3$): δ 8.88 (s, 1H, NH), 6.81–7.87 (m, 19H, Ar-H), 3.77 (s, 3H, –OCH_3). Anal. Calcd for $C_{30}H_{23}N_3O_3$: C, 76.09; H, 4.90; N, 8.87. Found: C, 75.90; H, 5.03; N, 8.84.

4.1.1.4. 4-[(4-Benzoyl-1,5-diphenyl-1*H*-3-pyrazolyl)carbonyl] aminobenzoic acid 4d. Compound **4d** was prepared according to the general procedure above in 60% yield; mp, 286–288 °C; IR (ν cm^{-1}): 2700–3364 (br, OH and NH), 1690 (br, C=O, carboxylic and C=O of benzoyl), 1661 (C=O, amide), and 1594 (C=N); ^{1}H NMR (60 MHz, $CDCl_3$): δ 10.00 (br s, COOH), 9.30 (br s, 1H, NH) and 7.5–8.8 (m, 19H, Ar-H). FAB-MS: m/z 488 (M+1). Anal. Calcd for $C_{30}H_{21}N_3O_4$: C, 73.91; H, 4.34; N, 8.62. Found: C, 73.40; H, 4.51; N, 8.59.

4.1.1.5. 4-Benzoyl-1,5-diphenyl-*N*{4-[(pyrimidin-2-yl-amino)sulfonyl]phenyl}-1*H*-pyrazole-3-carboxamide 4e. Compound **4e** was prepared according to the general procedure above in 63.0% yield; mp, 255–256 °C; ^{1}H NMR (60 MHz, $CDCl_3$): δ 11.00 (br s, NH), 9.35 (br s, 1H, NH), 7.60–8.65 (m, 19H, Ar-H). Anal. Calcd for $C_{33}H_{24}N_6O_4S$: C, 65.99; H, 4.03; N, 13.99. Found: C, 66.31; H, 4.13; N, 13.61.

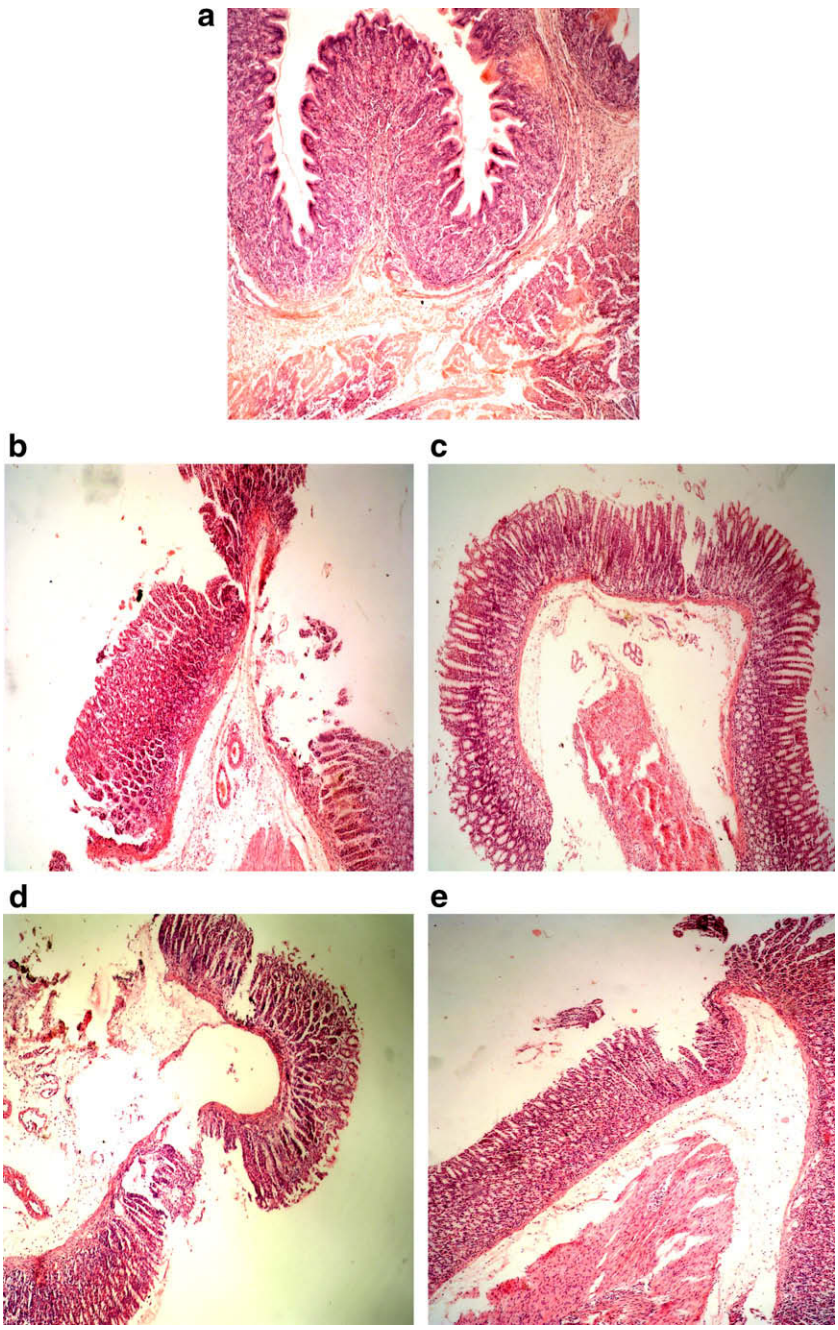


Figure 2. (a) Photomicrograph of the mucosa of fundic stomach of control. (b) Photomicrograph of the mucosa of fundic stomach of compound **4a**. (c) Photomicrograph of the mucosa of fundic stomach of compound **5a**. (d) Photomicrograph of the mucosa of fundic stomach of compound **4g**. (e) Photomicrograph of the mucosa of fundic stomach of compound **10**.

4.1.1.6. *N*-(4-Acetylphenyl)-4-benzoyl-1,5-diphenyl-1*H*-3-pyrazolecarboxamide 4f. Compound **4f** was prepared according to the general procedure above in 65% yield; mp, 189–190 °C; IR (ν cm^{-1}): 3368 (NH), 1680 (C=O, acetyl), 1659 (br s, C=O, benzoyl) and (C=O, amide), and 1595 (C=N); ^{1}H NMR (60 MHz, $CDCl_3$): δ 9.75 (s, 1H, NH), 7.4–8.3 (m, 19H, Ar-H) and 2.5 (s, 3H, CH_3). Anal. Calcd for $C_{31}H_{23}N_3O_3$: C, 76.69; H, 4.77; N, 8.65. Found: C, 76.47; H, 4.52; N, 8.53.

4.1.1.7. (4-Benzoyl-1,5-diphenyl-1*H*-pyrazol-3-yl)(piperazin-1-yl)methanone 4g. Compound **4g** was prepared by a modification of the general procedure above where a solution of the acid chloride **3** in toluene was added in a dropwise manner to a solution of piperazine in toluene in an equimolar ratio to give the monoamide **4g**, mp, >300 °C; yield = 63%; IR (ν cm^{-1}): 3423 (NH), 1657 (C=O, benzoyl), 1629 (C=O, amide), and 1597 (C=N); ^{1}H NMR (200 MHz, DMSO-d_6): δ 7.18–7.70 (m, 15H, Ar-H), 2.80–3.92 (m, 9H, piperazine 8H + NH). Anal. Calcd for $C_{27}H_{24}N_4O_2$: C, 74.29; H, 5.54; N, 12.84. Found: C, 74.32; H, 5.54; N, 12.92.

4.1.2. General procedure for synthesis of the oximes 5a–e

A solution of hydroxylamine hydrochloride (0.347 g, 5 mmol) and triethylamine (0.505 g, 5 mmol) or pyridine (0.395 g, 5 mmol) in 15 mL absolute ethanol was added dropwise with stirring to the respective amide **4a–e** (1 mmol). The mixture was heated at reflux for 4–30 h and the solvent was evaporated under vacuum. To the cooled residue, 10 mL water was added, cooled in an ice bath and stirred until the oxime crystallized. The precipitated product

was filtered off and washed with little water, then dried. The crude product was recrystallized from the appropriate solvent to afford the oximes **5a–e** in yields ranging from 60% to 82%.

4.1.2.1. *N*-(4-Chlorophenyl)-4-[hydroxyimino(phenyl)methyl]-1,5-diphenyl-1*H*-3-pyrazolecarboxamide 5a. Compound **5a** was prepared using the general procedure above in 81% yield; mp, 116–118 °C (ethanol); time of the reaction is about 18 h using TEA as a base; IR (ν cm^{-1}): 3100–3600 (br, OH, oxime and NH), 1676 (C=O, amide) and 1595 (C=N). ^{1}H NMR (60 MHz, DMSO-d_6): δ 12.3 (br s, 1H, oximic OH, exchangeable with D_2O), 9.5 (br s, 1H, NH, exchangeable with D_2O) and 7.8–8.6 (m, 19H, Ar-H). MS: 478 (8.2%) ($M^{\cdot 2+}$–OH) 476 ($M^{\cdot +}$–OH) (30.7%), 350 (34.8%), 272 (10.4%), 180 (45.8%), 129 (12.4%), 127 (35.1%), 105 (33.4%), and 77 (100%). Anal. Calcd for: $C_{29}H_{21}ClN_4O_2{\cdot}H_2O$: C, 68.17; H, 4.54; N, 10.96. Found: C, 68.50; H, 4.58; N, 10.59.

4.1.2.2. *N*-(4-Methylphenyl)-4-[hydroxyimino(phenyl)methyl]-1,5-diphenyl-1*H*-3-pyrazolecarboxamide 5b. Compound **5b** was prepared using the general procedure above in 64% yield; mp, 187–189 °C (ethanol); time of reaction is about 18 h using TEA as a base; IR (ν cm^{-1}): 3100–3700 (br, OH, oxime and NH), 1682 (C=O, amide), and 1595 (C=N). ^{1}H NMR (60 MHz, DMSO-d_6): δ 9.9 (s, 1H, OH, exchangeable), 9.8 (s, 1H, NH), 7.1–8.9 (m, 19H, Ar-H) and 2.3 (s, 3H, CH_3); MS: 473.3 (100%) ($M^{+\cdot}$+H^+), 455.3 (32%) ($M^{+\cdot}$–OH), 351 (7%). Anal. Calcd for $C_{30}H_{24}N_4O_2{\cdot}0.4H_2O$: C, 75.11; H, 5.21; N, 11.68. Found: C, 75.25; H, 5.38; N, 11.21.

4.1.2.3. *N*-(4-Methoxyphenyl)-4-[hydroxyimino(phenyl)methyl]-1,5-diphenyl-1*H*-3-pyrazolecarboxamide 5c. Compound **5c** was prepared using the general procedure above in 63% yield; mp, 125–127 °C (ethanol); time of reaction is 4 h using pyridine as a base; IR (ν cm^{-1}): 2625–3500 (br, OH, oxime and NH), 1676 (C=O, amide), and 1598 (C=N). ^{1}H NMR (200 MHz, $CDCl_3$) δ 8.8 (s, 1H, OH), 8.6 (s, NH) and 6.7–7.5 (m, 19H, Ar-H). MS: 488 (23.3%) ($M^{+\cdot}$), 366 (40.6%), 350 (39.6%), 272 (11.2%), 123 (100%) and 77 (79%). Anal. Calcd for $C_{30}H_{24}N_4O_3{\cdot}1.6H_2O$: C, 69.65; H, 5.30; N, 10.83. Found: C, 69.69; H, 5.24; N, 11.07.

4.1.2.4. 4-[(4-[Hydroxyimino(phenyl)methyl]-1,5-diphenyl-1*H*-3-pyrazolylcarbonyl)- amino]benzoic acid 5d. Compound **5d** was prepared using the general procedure above in 60% yield; mp, 258–260 °C, (ethanol); time of reaction is 10 h using pyridine as a base; IR (ν cm^{-1}): 2500–3500 (br, OH oxime, OH carboxylic and NH), 1686 (C=O, acid), 1650 (C=O, amide) and 1600 (C=N). ^{1}H NMR (60 MHz, $CDCl_3$) δ 10.3 (s, OH oxime), 10.2 (s, NH) and 7.4–8.6 (m, 19H, Ar-H); MS: 485 (12.2%) ($M^{+\cdot}$–OH), 366 (23.8%), 348 (11.7%), 137 (44.2%) and 77 (100%). Anal. Calcd for $C_{30}H_{22}N_4O_4{\cdot}H_2O$: C, 69.22; H, 4.65; N, 10.76. Found: C, 69.41; H, 4.58; N, 10.51.

4.1.2.5. *N*-4-[(2-Pyrimidinylamino)sulfonyl]phenyl-4-[hydroxyimino(phenyl)methyl]-1,5-diphenyl-1*H*-3-pyrazolecarboxamide 5e. Compound **5e** was prepared using the general procedure above in 63% yield; mp, 192–193 °C (CH_2Cl_2/*n*-hexane); time of reaction about 30 h using pyridine as a base; IR (ν cm^{-1}): 2900–3500 (OH, oxime), 3300 (NH), 1670 (C=O, amide) and 1590 (C=N). ^{1}H NMR (60 MHz, $CDCl_3$). δ 12.2 (N*H*–SO_2), 8.5 (OH, oxime), 8.4 (N*H*–C=O) 7.1–8.2 (m, 22H, Ar-H); MS: 598 (9.4%) ($M^{+\cdot}$–OH), 535 (12.9%), 348 (60.6%), 185 (28.0%), 103 (10.3%) and 77 (100%). Anal. Calcd for $C_{33}H_{25}N_7O_4S{\cdot}0.5H_2O$: C, 63.45; H, 4.20; N, 15.70. Found: C, 63.74; H, 4.64; N, 15.68.

4.1.3. *N*-[4-(1-Hydroxyiminoethyl)phenyl]-4-benzoyl-1,5-diphenyl-1*H*-3-pyrazole- carboxamide 6

The oxime **6** was prepared from the intermediate **4f** by the previously described general procedure for synthesis of oximes **5a–e**; the time of the reaction is 3 h using TEA as a base and crystallization from methanol; mp = 236–238 °C; yield = 52%; IR (KBr, ν cm^{-1}): 3225–3370 (br, OH, oxime), 3368 (NH), 1670 (C=O, amide), and 1595 (C=N). ^{1}H NMR (60 MHz, $CDCl_3$). δ 9.7 (s, 1H, NH, exchangeable), 7.8–8.5 (m, 19H, Ar-H), 2.3 (s, 3H, CH_3). M.S (*m*/*z*) (%): 500 (19.1%) ($M^{+\cdot}$), 351 (100%), 150 (1.5%), 105 (28.9%), 103 (10.3%) and 77 (43.5%). Anal. Calcd for $C_{31}H_{24}N_4O_3{\cdot}0.5H_2O$: C, 73.07; H, 4.95; N, 11.00. Found: C, 73.33; H, 4.99; N, 11.13.

4.1.4. 2-Bromoethyl 4-benzoyl-1,5-diphenyl-1*H*-pyrazole-3-carboxylate 7

A mixture of the acid chloride **3** (0.386 g, 1 mmol) and 2-bromoethanol (0.248 g, 2 mmol) was heated at reflux in 10 mL dry toluene with a catalytic amount of pyridine for 3 h. After cooling the solution was acidified by adding 0.5 mol HCl, a crude solid was precipitated, filtered off and dried. The obtained crude product was purified by column chromatography using silica gel and dichloromethane as an eluent system to afford 0.20 g, (% yield = 49) of colourless needles of, mp = 142–144 °C; ^{1}H NMR (200 MHz, $CDCl_3$). δ 7.16–7.88 (m, 15H, Ar-H), 4.4 (t, 2H, CH_2), 3.2 (t, 2H, CH_2). Anal. Calcd for $C_{25}H_{19}BrN_2O_3$: C, 63.17; H, 4.03; N, 5.89. Found: C, 63.41; H, 4.29; N, 5.81.

4.1.5. 2-(Nitrooxy)ethyl 4-benzoyl-1,5-diphenyl-1*H*-pyrazole-3-carboxylate 8

A mixture of the ester **7** (0.474 g, 1 mmol), and $AgNO_3$ (0.676 g, 4 mmol) was heated at reflux in 15 mL acetonitrile overnight at 80 °C. The inorganic solid was filtered and discarded. The filtrate was evaporated till dryness, the residue was dissolved in 15 mL dichloromethane and washed with water (3 × 10 mL), brine (2 × 10 mL) and finally with water (2 × 10 mL). The organic layer was dried over anhydrous sodium sulfate, evaporated under vacuum and the obtained residue was recrystallized from absolute ethanol to give 0.10 g, (42%) of white needles, mp = 150–151 °C; ^{1}H NMR (200 MHz, $CDCl_3$). δ 7.2–7.9 (m, 15H, Ar-H), 4.4 (s, 4H, –CH_2CH_2–). MS (*m*/*z*) (%): 457 (20.8%) ($M^{+\cdot}$), 367 (1.62%), 352 (24.0%), 351 (72.7%), 291 (6.02%), 219 (4.7%), 180 (27%), 105 (68%) and 77 (100%). Anal. Calcd for $C_{25}H_{19}N_3O_6$: C, 65.64; H, 4.19; N, 9.19. Found: C, 66.24; H, 4.38; N, 8.84.

4.1.6. 1-4-[(4-Benzoyl-1,5-diphenyl-1*H*-3-pyrazolyl) carbonyl]piperazino}-2-chloro-1-ethanone 9

To an ice cooled stirred solution of the amide **4g** (1.833 g, 0.0042 mol) in dichloromethane. a solution of potassium carbonate (0.868 g, 0.0063 mol) in 100 mL water was added. Chloroacetyl chloride (0.519 g, 0.0046 mol) in 20 mL dichloromethane was added to this solution, chloroacetyl chloride (0.519 g, 0.0046 mol) in 20 mL dichloromethane was added in a dropwise manner while stirring over 30 min. The mixture was stirred for 2 h at 0 °C and 24 h at room temperature. The organic layer was separated and the aqueous layer was extracted with (2 × 30 mL) dichloromethane, the combined organic layer was washed with distilled water (3 × 20 mL). The organic layer was dried over anhydrous sodium sulfate and evaporated under vacuum. The obtained crude product was recrystallized from toluene to give 0.330 g of white crystals (20% yield); mp = 259–260 °C; ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.2–7.8 (m, 15H, Ar-H) and 2.5–4.0 (m, 10H, piperazine 8H + O=C–CH_2). FAB-MS: *m*/*z* 513 (M+1). Anal. Calcd for $C_{29}H_{25}ClN_4O_3$: C, 67.90; H, 4.91; N, 10.92. Found: C, 67.40; H, 4.74; N, 10.63.

4.1.7. 2-[4-(4-benzoyl-1,5-diphenyl-1*H*-pyrazole-3-carbonyl) piperazin-1-yl]-2-oxoethyl nitrate 10

The nitrate ester **10** was prepared from the amide **9** (0.30 g, 0.5 mmol) using the previously described procedure for synthesis

of the nitrate ester **8** to give the compound **10**; mp = 229–230 °C; yield (0.095 g, 30%). ^{1}H NMR (200 MHz, DMSO-d_6) δ 7.1–7.7 (m, 15H, Ar-H), 5.4 (s, 2H, CH_2–ONO_2) and 2.4–4 (m, 8H, piperazine). Anal. Calcd for $C_{29}H_{25}N_5O_6$: C, 64.56; H, 4.67; N, 12.98; Found: C, 64.62; H, 4.43; N, 13.12.

4.1.8. 4-Benzoyl-1,5-diphenyl-1*H*-pyrazole-3-carboxylic acid 2-hydroxyethyl ester 11

The ester **11** was prepared by reaction between equimolar mixture of the acid chloride **3** and ethylene glycol in a similar procedure to the ester **7**. The crude oily product was purified using silica gel column chromatography with dichloromethane/methanol in 9:1 ratio as eluent in (0.59 g, 55% yield) as oily product solidified upon standing.

^{1}H NMR (60 MHz, $CDCl_3$). δ 3.85–4.20 (m, 4H, $-CH_2CH_2-$); 4.80 (br s, 1H, OH), 8.0–8.4 (m, 15H, Ar-H).

4.2. Evaluation of nitric oxide release

A solution of the appropriate compound (20 μL of 1×10^{-4} mol in 100 mL DMSO) was added to 2 mL of 1:1 V/V mixture of 50 mM phosphate buffer (pH 7.4) and methanol containing (5×10^{-4} M) L-cysteine. After 1 h at 37C°, 1 mL of the reaction mixture was treated with 250 μL of Griess reagent. After 25 min, the absorbance was measured at 540 nm. Sodium nitrite standard solutions (10–50 nmol/mL) were used to construct the calibration curve.[24,30] The Griess reagent was prepared as follows: [sulfanilamide (4 g), *N*-naphthylethylenediamine dihydrochloride (0.2 g), 85% phosphoric acid (10 mL) in distilled water (final volume: 100 mL)].[24,30]

4.3. Investigation of the antibacterial activity

Compounds **4a**, **4c–f**, **5a**, **5c–e**, **6**, **7** and **8** were evaluated for their in vitro antibacterial activity using agar cup diffusion method.[25] Ciprofloxacin was used as a reference.

4.3.1. Materials and methods

The selected compounds were evaluated for antibacterial activity against *E. coli* C-600, *Klebsila pneumonia* (as Gram negative), *B. subitilis and S. aureus* NCTC 6571 (as Gram positive) using the agar cup diffusion method.[25]

4.3.2. Preparation of the media

All strains were cultured on sterile nutrient agar medium which was supplied by oxoid, and prepared according to the instructions of the manufactures. The media were molten on a steam bath inoculated with few drops of the culture of the specific microorganism and poured into sterile Petri dishes to form a layer of about 3–4 mm thickness. The layer was allowed to cool and harden, the under side of each plate was marked into approximately equal six sectors. With the aid of Wassrman tube, a single cup of about 10 mm diameter was cut in the centre of each sector to produce a total of six cups per dish. Five of these cups were devoted for the testing of the desired compounds, while the last one was left as a control guide for the solvent.

4.3.3. Preparation of the solutions of the test compounds

All compounds were first dissolved in DMF in a stock concentration of 1 mg/mL and kept at −20 °C until use. Two folds serial dilutions of each compound were performed from 32 to 512 μg/mL to determine its MIC for each strain.

4.3.4. Procedure

A constant volume of 0.1 mL of each of the prepared stock solutions was pipetted into the appropriate cup. Cultures were incubated for 24 h at 37 °C, and then the inhibition zone diameter was measured in mm. The minimal inhibitory concentration (MIC) in μg/mL was determined in μg/mL. The MIC was defined to be the intercept of the curve of logarithm concentrations versus diameter of the inhibition zones.

4.4. Screening of the anti-inflammatory activity

4.4.1. Carrageenan induced paw edema method

The anti-inflammatory activity of compounds **2**, **4a–g**, **5a–e** and **6–10** was screened according to the method described by Winter et al.[26] where a pedal inflammation in rat paws induced by subplanar injection of carrageenan suspension (1% w/v) into the right hind of the rats. Male albino rats 140–150 g were randomly divided into groups which consisted of 5 rats each. All the animals were left for three days in the laboratory for acclimatization before the day of experiment, and on the last day they were given water only. The thickness of rat paw was measured by a digital plethysmometer before and 30 min after carrageenan injection to detect the inflammation induced by carrageenan. Reference group was taken indomethacin orally at a dose 100 mg/mL. Control group received a vehicle (carboxymethylcellulose), while tested compounds **2**, **4a–g**, **5a–f** and **6–10** at dose of 100 mg/kg were taken orally to different groups of rats. The difference between the thicknesses of the two paws was taken as a measure of edema. The measurement was carried out at 0, 1, 2, 3, 4 and 5 h after administration of the tested compounds, reference and vehicle.

4.5. Investigation of the gastric protective activity

After measuring the anti-inflammatory activity the rats were killed by an overdose of ether. All experiments were performed at the same time of the day to avoid variations due to diurnal rhythms of putative regulators of gastric functions. The stomachs were removed and opened along the greater curvature. The stomachs were washed with ice-cold saline and scored for macroscopic gross mucosal lesions.[31]

4.6. Histopathological investigation

The histological slides were prepared according to the reported procedures[29] for examination of ulcers under light microscope.

4.6.1. Procedures

Identify site of the slide on which the section was applied by scratching wax around section with a needle. Dewax hydrated sections by using graded alcohols to water. Slides were stained with haematoxylin for 5–7 min, washed with tap water until sectioning for 5 min. and immersed for 5–10 s in solution of (1% HCl in 70% alcohol), then washed well with tap water for 10–15 min. followed by staining with 1% Eosin for 10 min, washed with running tap water for 1–5 min. The slide was then dehydrated using alcohols, cleaned by using xylene, covered by glass cover using Canda balsam then examined under microscope.

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