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Full Paper

Some Novel Mannich Bases of 5-(3,4-Dichlorophenyl)-1,3,4-oxadiazole-2(3*H*)-one and Their Anti-Inflammatory Activity

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Non-steroidal anti-inflammatory drugs (NSAIDs), which are widely used for the treatment of rheumatic arthritis, pain, and many different types of inflammatory disorders, cause serious gastrointestinal (GI) side effects. The free carboxylic acid group existing on their chemical structure is correlated with GI toxicity related with all routine NSAIDs. Replacing this functional group with the 1,3,4-oxadiazole bioisostere is a generally used strategy to obtain an anti-inflammatory agent devoid of GI side effects. In the present work, a novel group of 5-(3,4-dichlorophenyl)-1,3,4-oxadiazole-2(3*H*)-one Mannich bases were synthesized and characterized on the basis of IR, ^{1}H NMR, and elemental analysis results. The target compounds were first tested for cytotoxicity to determine a non-toxic concentration for anti-inflammatory screening. Anti-inflammatory effects of the compounds were evaluated by *in vitro* lipopolysaccharide (LPS)-induced NO production and *in vivo* carrageenan footpad edema with ulcerogenic profile. In LPS-induced RAW 264.7 macrophages, most of the compounds showed inhibitory activity on nitrite production while compounds **5a**, **5h**, and **5j** exhibited the best profiles by suppressing the NO production. To evaluate the *in vivo* anti-inflammatory potency of the compounds, the inflammatory response was quantified by increment in paw size in the carrageenan footpad edema assay. The anti-inflammatory data scoring showed that compounds **5a–d**, **5g**, and **5j**, at the dose of 100 mg/kg, exhibited anti-inflammatory activity, which for compound **5g** was comparable to that of the reference drug indomethacin with 53.9% and 55.5% inhibition in 60 and 120 min, respectively.

Keywords: 1,3,4-Oxadiazole-2(3*H*)-one / Anti-inflammatory activity / Mannich reaction / Piperidine

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Introduction

Nitrogen and oxygen containing heterocyclic compounds are well known as important building blocks in organic and medicinal chemistry [1]. Especially, 1,3,4-oxadiazole is widely

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studied by the chemists due to its pharmacophore feature for ligand binding [2, 3]. A great number of pharmacological properties have been associated with this small but important core structure such as anti-inflammatory [4–7], antimicrobial [8–11], antitubercular [12–14], and anticancer [15–19]. Non-steroidal anti-inflammatory drugs (NSAIDs) are a good choice to use for the treatment of pain, fever, and inflammation, particularly arthritis [20, 21]. Principal symptoms of inflammation such as erythema, raised temperature, hyperalgesia, and pain which are due to reaction of tissue harm, are caused by aggregation of leucocytes and serum ingredients [22]. The pharmacological activity of NSAIDs is related to the suppression of prostaglandin (PG) biosynthesis from arachidonic acid by inhibiting the cyclooxygenase (COX) enzymes. Prostaglandins that cause fever, pain, and inflammation are formed by cyclooxygenase-2 (COX-2) isoenzyme whereas others regulate beneficial gastrointestinal cytoprotection due to cyclooxygenase-1 (COX-1) mechanism [23]. A prominent side effect on chronic use of NSAIDs is generally attributed to two main factors: decreased tissue prostaglandin production and local irritation by the direct contact of carboxylic acid (–COOH) moiety of NSAIDs with GI mucosal cells [24]. To overcome this problem, synthetic approaches based upon chemical modifications of free carboxylic acid group existing on NSAIDs is used to improve their safety profile. The literature survey revealed that replacing the free carboxylic functional group with 1,3,4-oxadiazole enhances the anti-inflammatory activity and decreases the gastric upset [25–27]. Furthermore, oxadiazole nucleus enhances the interaction by formation of numerous hydrogen bonds with cyclooxygenase receptor when it is compared with carboxylic acid moiety [28, 29].

Nitric oxide (NO) is an important regulator in inflammatory diseases. It gives an anti-inflammatory effect under normal conditions whereas it causes tissue destruction and cellular death in high amounts. NO increases in several inflammatory diseases such as asthma and arthritis. Therefore, inhibition of NO production is important strategy for the screening of anti-inflammatory agents [30–32].

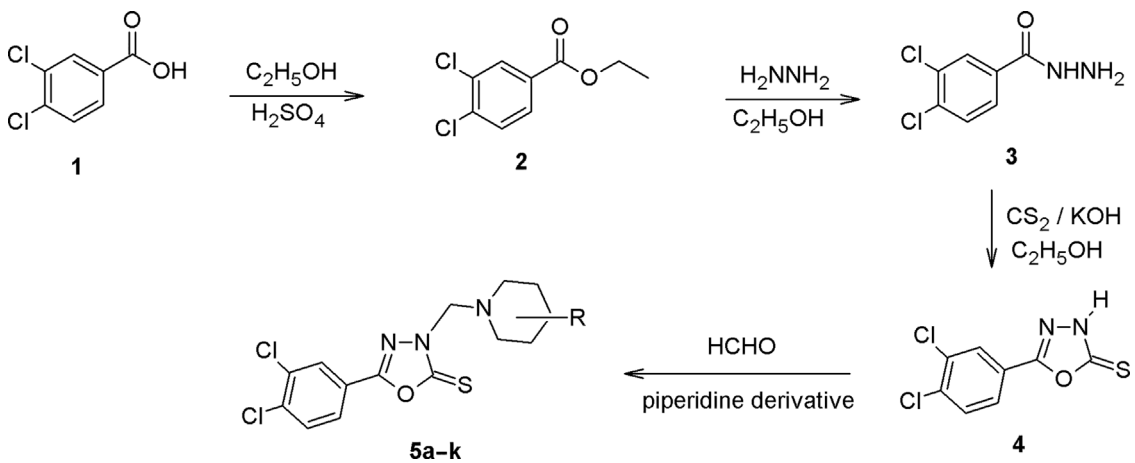
In view of these observations and in continuation of our research studies on the synthesis of 5-aryl-1,3,4-oxadiazole-2(3*H*)-one Mannich bases [33, 34], we report herein the synthesis of some novel 3,5-disubstituted-1,3,4-oxadiazole derivatives which have been found to posses promising anti-inflammatory activity with significantly reduced ulcerogenic effect.

Results and discussion

Chemistry

The preparation of target compounds (**5a–j**) is described in Scheme 1. The key intermediate 5-(3,4-dichlorophenyl)-1,3,4-oxadiazol-2(3*H*)-one (**4**) was synthesized in three steps. Esterification of the 3,4-dichlorobenzoic acid (**1**) with ethanol and concentrated sulfuric acid afforded the corresponding ester (**2**). The aroyl hydrazide (**3**) was obtained by the reaction of ethyl 3,4-dichlorobenzoate (**2**) with hydrazine hydrate monohydrate (85%) in ethanol. Then the treatment of hydrazide (**3**) with 1,1-carbonyldiimidazole (CDI) in the presence of triethylamine (TEA) and tetrahydrofuran (THF) by stirring at room temperature gave the key intermediate (**4**). The 3,5-disubstituted-1,3,4-oxadiazole-2(*3H*)-one derivatives (**5a–j**) were prepared via Mannich reaction of 5-(3,4-dichlorophenyl)-1,3,4-oxadiazol-2(*3H*)-one (**4**), substituted piperidines and formaldehyde in ethanol [33].

All of the compounds gave satisfactory analytical and spectroscopic data, which were in full accordance with their depicted structures. IR spectra of the synthesized compounds are similar to the IR values of which were stated in the literature [35, 36]. For the compounds **5a–j**, no absorption band was detected at 3100–3400 cm^{-1}, indicating the absence



Scheme 1. Synthesis of 5-(3,4-dichlorophenyl)-1,3,4-oxadiazole-2(3*H*)-one Mannich bases.

of an NH group as an evidence for the substitution reaction to 5-(3,4-dichlorophenyl)-1,3,4-oxadiazol-2(3*H*)-one with substituted piperidine. In the ^{1}H NMR spectra of all compounds, the methylene protons representing the Mannich base formation were seen at about 4.70–4.80 ppm as a singlet. The protons of the 3,4-dichlorophenyl group were seen approximately at 7.95 (d, $J = 2$ Hz, 1H, H^2), 7.65 (dd, $J = 8.2$, 2 Hz, 1H, H^5), and 7.55 (d, $J = 8.8$ Hz, 1H, H^6) ppm, respectively. As $H^{3'}$ and $H^{5'}$ protons of the piperidine ring are overlapped and seen as a triplet peak at 1.23–1.86 ppm, $H^{2'}$ and $H^{6'}$ protons are seen at 2.46–3.47 ppm likewise.

In vitro anti-inflammatory activity

Cell cytotoxicity

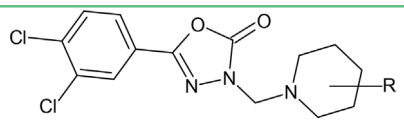
Before the screening studies for inhibition of NO production, it was essential to carry out cytotoxicity testing in order to determine safe and non-toxic concentrations of every compound. Therefore, compounds were analyzed for their cytotoxic effects on RAW 264.7 macrophages by using the MTT assay. Indomethacin, which is one of the most effective anti-inflammatory agents, was used as the reference drug in all biological studies. It was shown that IC_{50} values of the tested compounds were higher than 100 μM (Table 1). According to the results, in the series of ten compounds, screening at 100 μM dose level is determined to be safe for NO production inhibition and data recorded for this dose will not be due to cytotoxicity against RAW 264.7 cells.

Nitrite assay

Anti-inflammatory activity of the tested compounds (**5a–j**) were evaluated by measuring NO levels in LPS-stimulated macrophage cells [37]. Every compound was tested at 100 μM concentration which would not cause any cytotoxic effect at this dose concentration (Fig. 1). Among the tested compounds, **5a–c**, **5g–j** significantly decreased nitrite production while the strongest inhibition with **5a**, **5h**, and **5j** was almost the same as with the reference drug indomethacin.

The phenyl substituted and carbonyl carrying compounds (**5a**, **5h–j**) were more active derivatives than the other bulky piperidine analogs as NO production inhibitors. Furthermore, benzyl and morpholine containing compounds were inactive both in *in vivo* and *in vitro* anti-inflammatory activity screening. This type of substitution in the core structure might not provide the proper orientation at the receptor site and lead to loss of potency.

Table 1. Structures of the target compounds and their IC_{50} values against RAW 264.7 macrophage cell viability.



Compound	R	IC_{50} (μM)
5a	4-Phenyl	146.35 ± 3.94
5b	4-Hydroxy-4-phenyl	150.66 ± 7.56
5c	4-Acetyl-4-phenyl	140.64 ± 5.45
5d	4-Cyano-4-phenyl	151.07 ± 4.64
5e	4-Benzyl	147.60 ± 4.91
5f	4-(Morpholin-4-yl)	146.38 ± 3.45
5g	4-(Piperidin-4-yl)	152.17 ± 9.62
5h	3-Carboxylic acid	127.16 ± 4.89
5i	4-Ethyloxycarbonyl	141.33 ± 4.47
5j	2-Ethyloxycarbonyl	146.05 ± 0.45
Indomethacin	–	>200

In vivo anti-inflammatory activity

Carrageenan footpad edema

The anti-inflammatory activity of the synthesized compounds was evaluated using carrageenan-induced rat paw edema assay. Also, ulcerogenic effect on acute administration was studied to assess the safety of the compounds. The inhibition of edema was measured after 60, 120, 180, and 240 min from the administration of the compounds. The anti-inflammatory and ulcerogenic results are given in Table 2.

Results revealed that compounds **5a–d**, **5g**, and **5j** showed anti-inflammatory profile with meaningful statistical results. It was also seen that substitution on fourth position of piperidine moiety could change the anti-inflammatory activity with variable edema inhibition percentages (32–55.5%). In general, phenyl-substituted analogs were more potent than the corresponding carbonyl-substituted ones. Although these carboxylic acid and ester derivatives were found active in *in vitro* anti-inflammatory NO assay, absence of activity in carrageenan footpad edema model except compound **5j** might be because of rapid metabolism of carboxylic acid and ester derivatives. The compound **5g** having piperidine moiety at fourth position of piperidine ring showed the most promising inhibition values, 53.9, 55.5, 41.2, and 40.9%, respectively, in 60–240 min examination period of swelling

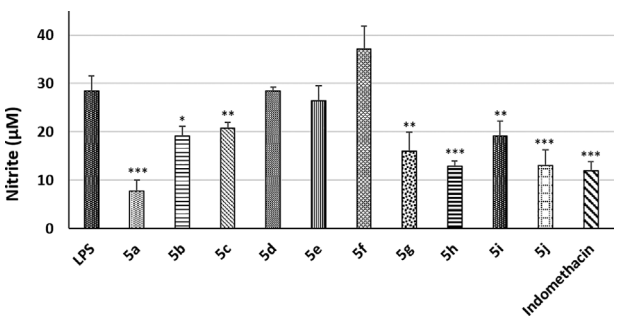


Figure 1. The amount of nitrite in LPS-induced RAW 264.7 macrophages. Results are expressed as the mean ± SD ($n = 3$). $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ versus the LPS-stimulated group.

Table 2. Results of the carrageenan-induced paw edema test and incidence of gastrointestinal lesions in rats.

Compound	Anti-inflammatory activity swelling in thickness (mm) ± SEM (inhibition %)				Ratio of ulceration[a)]
	60 min	120 min	180 min	240 min	
Control	2.01 ± 0.15	2.53 ± 0.19	2.90 ± 0.19	3.02 ± 0.22	0/6
5a	1.39 ± 0.24	1.49 ± 0.19 (41.1)*	1.96 ± 0.14 (32.6)*	2.05 ± 0.14 (32.0)*	0/6
5b	1.22 ± 0.24	1.47 ± 0.28 (42.1)*	1.67 ± 0.25 (42.6)*	1.74 ± 0.37 (42.3)*	0/6
5c	1.07 ± 0.12 (46.9)*	1.58 ± 0.25 (37.5)*	1.93 ± 0.36	2.05 ± 0.36	0/6
5d	0.99 ± 0.33 (51.1)*	1.41 ± 0.32 (44.2)*	2.28 ± 0.36	2.33 ± 0.27	0/6
5e	1.50 ± 0.26	2.16 ± 0.21	2.74 ± 0.25	2.78 ± 0.19	0/6
5f	1.81 ± 0.17	2.36 ± 0.15	2.77 ± 0.19	2.70 ± 0.30	0/6
5g	0.93 ± 0.21 (53.9)**	1.13 ± 0.18 (55.5)**	1.71 ± 0.20 (41.2)*	1.78 ± 0.15 (40.9)**	1/6
5h	1.77 ± 0.30	2.45 ± 0.26	2.82 ± 0.27	2.83 ± 0.29	0/6
5i	1.83 ± 0.26	2.09 ± 0.30	2.85 ± 0.38	2.76 ± 0.36	0/6
5j	1.76 ± 0.33	1.72 ± 0.22 (32.3)*	1.84 ± 0.16 (36.7)*	1.94 ± 0.16 (35.5)*	0/6
INDO	1.01 ± 0.15 (49.8)**	1.16 ± 0.28 (54.3)**	1.40 ± 0.26 (51.8)**	1.46 ± 0.27 (51.7)**	2/6

[a)] Number of rats showing ulcer/total number of rats. * $p < 0.05$, ** $p < 0.01$ compared to control group.

in thickness of foot paw edema. On the other hand, replacement of this ring by morpholine resulted in loss of activity. The compounds which were screened for their *in vivo* anti-inflammatory activity were further tested for their ulcerogenic liability. Results showed that all the compounds (**5a–j**) exhibited a safe profile compared to that of indomethacin.

Conclusion

In this study, a new series of Mannich bases of 5-(3,4-dichlorophenyl)-1,3,4-oxadiazole-2(3*H*)-one was synthesized (**5a–j**), tested for their biological effects as inhibitors of LPS-induced NO production in RAW 264.7 macrophages and evaluated for their anti-inflammatory profile in carrageenan-induced rat paw edema assay. In LPS-induced macrophages, most of the compounds suppressed the production of NO which can be useful for targeting the treatment of anti-inflammatory diseases. Related to these results, compounds were tested in a simple and routine animal model for evaluation of activity at the site of inflammation. The improvement of edema in the rat hind paw and suppression of the release of NO following the injection of carrageenan was observed with decrease of swelling in thickness. Calculated inhibition % values showed that anti-inflammatory activity profile was generally parallel to *in vivo* NO assay except for the carboxylic acid and ester carrying derivative which can rapidly undergo biotransformation.

As a conclusion, the current study showed that the synthesized compounds could be used as a part of template for future development through modification and derivatization to design more potent anti-inflammatory compounds that carry 1,3,4-oxadiazole core structure. In order to produce a rational quantitative structure–activity relationship (QSAR) mapping, future synthesis of similar derivatives will take place to create a larger set of compounds.

Experimental

Chemistry

General

All the chemicals used for the synthesis of the compounds were purchased from Sigma–Aldrich (Germany). Melting points (°C) were determined by using a Mettler Toledo FP62 capillary melting point apparatus (Mettler-Toledo, Greifensee, Switzerland) and are uncorrected. Infrared spectra were recorded on a Perkin-Elmer Spectrum One series FTIR apparatus (Version 5.0.1) (Perkin-Elmer, Norwalk, CT, USA), using potassium bromide pellets, the frequencies were expressed in cm^{-1}. The ^1H NMR spectra were recorded with a Varian Mercury-400 FT-NMR spectrometer (Varian Inc., Palo Alto, CA, USA), using tetramethylsilane as the internal reference, $CDCl_3$ as solvent, the chemical shifts were reported in parts per million (ppm). Coupling constants (*J*) were given in hertz (Hz). Elemental analyses were performed on LECO 932 CHNS (Leco-932, St. Joseph, MI, USA) instrument. All the compounds gave C, H, and N analysis within ±0.4% of the theoretical values.

The InChI codes of the investigated compounds together with some biological activity data are provided as Supporting Information.

Synthesis of 5-(3,4-dichlorophenyl)-1,3,4-oxadiazol-2(3H)-one (4)

To a 0°C solution of 3,4-dichlorobenzohydrazide (2.2 mmol, 0.451 g) and triethylamine (TEA) (2.2 mmol) in THF (10 mL), 1,1-carbonyldiimidazole (CDI) was added. The resulting

mixture was stirred for 20 h at room temperature and concentrated *in vacuo*. The residue was dissolved in diethyl ether (15 mL), washed with 2 M hydrochloric acid (5 mL) and saturated aqueous sodium bicarbonate, and then dried with sodium sulfate. Filtration and concentration *in vacuo* gave compound **4**, which was recrystallized from ethanol/water [33].

*Synthesis of 5-(3,4-dichlorophenyl)-3-[(4-substituted-piperidine-1-yl)methyl]-1,3,4-oxadiazol-2(3H)-ones (**5a–j**)*
To a solution of 5-(3,4-dichlorophenyl)-1,3,4-oxadiazol-2(3*H*)-one **4** (1 mmol, 0.231 g) in ethanol (10 mL), a mixture of formaldehyde (1.5 mmol) and piperidine derivative (1 mmol) in ethanol was added by stirring. After complete addition, the mixture was refluxed for 4 h. The solution was precipitated by cooling, the formed compound was filtered and crystallized with ethanol/water.

*5-(3,4-Dichlorophenyl)-3-[(4-phenylpiperidin-1-yl)-methyl]-1,3,4-oxadiazol-2(3H)-one (**5a**)*
White solid; yield: 78%; m.p. 153.4°C; IR (ν_{max}, KBr pellets, cm^{-1}): 3059–2806 (C–H), 1773 (C=O), 1604 (C=N), 1245 (C–O); ^{1}H NMR ($CDCl_3$) δ_H: 1.81 (2H, t, $J = 12.6$ Hz, piperidine H_3, H_5 equatorial), 1.89 (2H, d, $J = 10.4$ Hz, piperidine H_3, H_5 axial), 2.42–2.48 (1H, m, piperidine H_4), 2.59 (2H, t, $J = 11.6$ Hz, piperidine H_2, H_6 equatorial), 3.17 (2H, d, $J = 11.6$ Hz, piperidine H_2, H_6 axial), 4.77 (2H, s, methylene), 7.18–7.12 (3H, m, phenyl H_2, H_4, H_6), 7.28–7.31 (2H, m, phenyl H_3, H_5), 7.57 (1H, d, $J = 8.4$ Hz, dichlorophenyl H_6), 7.70 (1H, dd, $J = 8$, $J' = 2$ Hz, dichlorophenyl H_5), 7.97 (1H, d, $J = 2$ Hz, dichlorophenyl H_2). Anal. calcd. for $C_{20}H_{19}Cl_2N_3O_2$: C, 59.42; H, 4.74; N, 10.39. Found: C, 59.36; H, 4.56; N, 10.56.

*5-(3,4-Dichlorophenyl)-3-[(4-hydroxy-4-phenylpiperidin-1-yl)methyl]-1,3,4-oxadiazol-2(3H)-one (**5b**)*
White solid; yield: 75%; m.p. 194.6°C; IR (ν_{max}, KBr pellets, cm^{-1}): 3540 (O–H), 3094–2836 (C–H), 1768 (C=O), 1604 (C=N), 1247 (C–O). ^{1}H NMR ($CDCl_3$) δ_H: 1.81 (2H, d, $J = 14$ Hz, piperidine H_3, H_5 axial), 2.12–2.20 (2H, m, piperidine H_3, H_5 equatorial), 2.96 (4H, d, $J = 8.4$ Hz, piperidine H_2, H_6), 4.78 (2H, s, methylene), 7.28 (1H, d, $J = 7.2$ Hz, phenyl H_4), 7.37 (2H, t, $J = 7.6$ Hz, phenyl H_3, H_5), 7.50 (2H, d, $J = 8$ Hz, phenyl H_2, H_6), 7.57 (1H, d, $J = 8.4$ Hz, dichlorophenyl H_6), 7.69 (1H, dd, $J = 8.4$, $J' = 2$ Hz, dichlorophenyl H_5), 7.97 (1H, d, $J = 2$ Hz, dichlorophenyl H_2). Anal. calcd. for $C_{20}H_{19}Cl_2N_3O_3$: C, 57.15; H, 4.56; N, 10.00. Found: C, 56.77; H, 4.61; N, 10.07.

*5-(3,4-Dichlorophenyl)-3-[(4-acetyl-4-phenylpiperidin-1-yl)methyl]-1,3,4-oxadiazol-2(3H)-one (**5c**)*
White solid; yield: 52%; m.p. 167.5°C; IR (ν_{max}, KBr pellets, cm^{-1}): 3078–2819 (C–H), 1776 (C=O), 1698 (C=O, acetyl), 1606 (C=N), 1252 (C–O). ^{1}H NMR ($CDCl_3$) δ_H: 1.88 (3H, s, $–CH_3$), 2.06 (2H, t, $J = 14.8$ Hz, piperidine H_3, H_5 axial), 2.50 (2H, d, $J = 12.4$ Hz, piperidine H_3, H_5 equatorial), 2.68 (2H, t, $J = 12$ Hz, piperidine H_2, H_6 axial), 2.92–2.96 (2H, m, piperidine H_2, H_6 equatorial), 4.68 (2H, s, methylene), 7.27–7.29 (3H, m, phenyl H_3, H_4, H_5), 7.32–7.36 (2H, m, phenyl H_2, H_6), 7.56 (1H, d, $J = 8.4$ Hz, dichlorophenyl H_6), 7.66 (1H, dd, $J = 8.4$, $J' = 2$ Hz, dichlorophenyl H_5), 7.93 (1H, d, $J = 2$ Hz, dichlorophenyl H_2). Anal. calcd. for $C_{22}H_{21}Cl_2N_3O_3$: C, 59.20; H 4.74; N, 9.41. Found: C, 59.45; H 4.86; N, 9.55.

*5-(3,4-Dichlorophenyl)-3-[(4-cyano-4-phenylpiperidin-1-yl)methyl]-1,3,4-oxadiazol-2(3H)-one (**5d**)*
White solid; yield: 61%; m.p. 128.9°C; IR (ν_{max}, KBr pellets, cm^{-1}): 3083–2827 (C–H), 2238 (C≡N), 1774 (C=O), 1609 (C=N), 1254 (C–O). ^{1}H NMR ($CDCl_3$) δ_H: 2.12–2.15 (4H, m, piperidine H_3, H_5), 2.98 (2H, t, $J = 10$ Hz, piperidine H_2, H_6 axial), 3.18 (2H, d, $J = 12.4$ Hz, piperidine H_2, H_6 equatorial), 4.77 (2H, s, methylene), 7.34–7.36 (3H, m, phenyl H_3, H_4, H_5), 7.50 (2H, d, $J = 8.4$ Hz, phenyl H_2, H_6), 7.58 (1H, d, $J = 8$ Hz, dichlorophenyl H_6), 7.70 (1H, dd, $J = 8$, $J' = 2$ Hz, dichlorophenyl H_5), 7.97 (1H, d, $J = 2$ Hz, dichlorophenyl H_2). Anal. calcd. for $C_{22}H_{21}Cl_2N_3O_3$: C, 58.75; H 4.23; N, 13.05. Found: C, 57.99; H 4.07; N, 12.96.

*5-(3,4-Dichlorophenyl)-3-[(4-benzylpiperidin-1-yl)methyl]-1,3,4-oxadiazol-2(3H)-one (**5e**)*
White solid; yield: 75%; m.p. 134.6°C; IR (ν_{max}, KBr pellets, cm^{-1}): 3092–2806 (C–H), 1777 (C=O), 1607 (C=N), 1248 (C–O). ^{1}H NMR ($CDCl_3$) δ_H: 1.28 (2H, qd, $J = 12$ Hz, piperidine H_3, H_5 axial), 1.45–1.56 (1H, m, piperidine H_4), 1.67 (2H, d, $J = 13.2$ Hz, piperidine H_3, H_5 equatorial), 2.39 (2H, t, $J = 12.0$ Hz, piperidine H_2, H_6 axial), 2.52 (2H, d, $J = 7.2$ Hz, benzyl $–CH_2$), 3.01 (2H, d, $J = 11.6$ Hz, piperidine H_2, H_6 equatorial), 4.70 (2H, s, methylene), 7.11 (2H, d, $J = 6.8$ Hz, benzyl H_2, H_6), 7.15–7.27 (3H, m, benzyl H_3, H_4, H_5), 7.55 (1H, d, $J = 8.8$ Hz, dichlorophenyl H_6), 7.66 (1H, dd, $J = 8$, $J' = 2$ Hz, dichlorophenyl H_5), 7.93 (1H, d, $J = 2$ Hz, dichlorophenyl H_2). Anal. calcd. for $C_{21}H_{21}Cl_2N_3O_2$: C, 60.30; H, 5.06; N, 10.05. Found: C, 60.22; H, 5.43; N, 10.17.

*5-(3,4-Dichlorophenyl)-3-{[4-(morpholin-4-yl)piperidin-1-yl]methyl}-1,3,4-oxadiazol-2(3H)-one (**5f**)*
White solid; yield: 59%; m.p. 134.6°C; IR (ν_{max}, KBr pellets, cm^{-1}): 3097–2821 (C–H), 1786 (C=O), 1609 (C=N), 1258 (C–O). ^{1}H NMR ($CDCl_3$) δ_H: 1.52–1.59 (2H, m, piperidine H_3, H_5 axial), 1.86 (2H, d, $J = 12.8$ Hz, piperidine H_3, H_5 equatorial), 2.11–2.16 (1H, m, piperidine H_4), 2.46 (2H, t, $J = 11.6$ Hz, piperidine H_2, H_6 axial), 2.53 (4H, t, $J = 4.8$ Hz, morpholine H_3, H_5), 3.10 (2H, d, $J = 11.6$ Hz, piperidine H_2, H_6 equatorial), 3.71 (4H, t, $J = 4.4$ Hz, morpholine H_3, H_5), 4.71 (2H, s, methylene), 7.56 (1H, d, $J = 8.4$ Hz, dichlorophenyl H_6), 7.68 (1H, dd, $J = 8.8$, $J' = 2$ Hz, dichlorophenyl H_5), 7.95 (1H, d, $J = 2$ Hz, dichlorophenyl H_2). Anal. calcd. for $C_{18}H_{22}Cl_2N_4O_3$: C, 52.31; H, 5.37; N, 13.56. Found: 52.26; H, 5.52; N, 13.92.

*5-(3,4-Dichlorophenyl)-3-{[4-(piperidin-4-yl)piperidin-1-yl]-methyl}-1,3,4-oxadiazol-2(3H)-one (**5g**)*
White solid; yield: 63%; m.p. 165.6°C; IR (ν_{max}, KBr pellets, cm^{-1}): 3091–2816 (C–H), 1781 (C=O), 1611 (C=N), 1253 (C–O).

^{1}H NMR ($CDCl_3$) δ_H: 1.22–1.25 (2H, m, piperidinyl H_4), 1.42–1.46 (2H, m, piperidinyl H_2, H_6 axial), 1.54–1.64 (4H, m, piperidinyl H_3, H_5), 1.84 (2H, d, $J = 12$ Hz, piperidinyl H_2, H_6 equatorial), 2.19–2.25 (1H, m, piperidine H_4), 2.41 (2H, t, $J = 12$ Hz, piperidine H_2, H_6 axial), 2.50 (4H, t, $J = 4.8$ Hz, piperidine H_3, H_5), 3.09 (2H, d, $J = 11.6$ Hz, piperidine H_2, H_6 equatorial), 4.70 (1H, s, methylene), 5.30 (1H, s, methylene), 7.56 (1H, dd, $J = 8$, $J' = 2.4$ Hz, dichlorophenyl H_6), 7.67–7.69 (1H, m, dichlorophenyl H_5), 7.94 (1H, d, $J = 2$ Hz, dichlorophenyl H_2). Anal. calcd. for $C_{19}H_{24}Cl_2N_4O_2$: C, 55.48; H, 5.88; N, 13.62. Found: C, 54.07; H, 6.03; N, 13.65.

*1-{[5-(3,4-Dichlorophenyl)-2-oxo-1,3,4-oxadiazol-3(2H)-yl]-methyl}piperidine-3-carboxylic acid (**5h**)*
White solid; yield: 58%; m.p. 171.3°C. IR (ν_{max}, KBr pellets, cm^{-1}): 3100 (OH), 2950–2837 (C–H), 1796 (C=O, RCOOH), 1713 (C=O), 1614 (C=N), 1244, 1199 (C–O). ^{1}H NMR ($CDCl_3$) δ_H: 1.63–1.68 (1H, m, piperidine H_5 axial), 1.75–1.79 (1H, m, piperidine H_5 equatorial), 2.42 (2H, t, $J = 12.4$ Hz, piperidine H_4), 2.50–2.56 (2H, m, piperidine H_6 axial, H_3), 2.81 (1H, d, $J = 11.2$ Hz, piperidine H_2 axial), 3.02 (1H, dd, $J = 11.2$, $J' = 3.2$ Hz, piperidine H_6 equatorial), 3.42–3.47 (1H, m, piperidine H_2 equatorial), 4.66 (2H, s, methylene), 7.77 (1H, dd, $J = 8.4$, $J' = 2$ Hz, dichlorophenyl H_6), 7.83 (1H, d, $J = 8.8$ Hz, dichlorophenyl H_5), 7.95 (1H, d, $J = 2$ Hz, dichlorophenyl H_2). Anal. calcd. for $C_{15}H_{15}Cl_2N_3O_4$: C, 48.40; H, 4.06; N, 11.29. Found: C, 47.43; H, 4.30; N, 10.84.

*Ethyl 1-{[5-(3,4-dichlorophenyl)-2-oxo-1,3,4-oxadiazol-3(2H)-yl]methyl}piperidine-4-carboxylate (**5i**)*
White solid; yield: 54%; m.p. 96.9°C; IR (ν_{max}, KBr pellets, cm^{-1}): 3068–2772 (C–H), 1760 (C=O, RCOOR'), 1732 (C=O), 1613 (C=N), 1213 (C–O). ^{1}H NMR ($CDCl_3$) δ_H: 1.24 (3H, t, $J = 10.8$ Hz, $–CH_3$), 1.73–1.80 (2H, m, piperidine H_3, H_5 axial), 1.94 (2H, dd, $J = 8.8$, $J' = 3.2$ Hz, piperidine H_3, H_5 equatorial), 2.20–2.26 (1H, m, piperidine H_4), 2.49 (2H, t, $J = 11.2$ Hz, piperidine H_2, H_6 axial), 3.04 (2H, d, $J = 12$ Hz, piperidine H_2, H_6 equatorial), 4.12 (2H, q, $COO–CH_2–$), 4.71 (2H, s, methylene), 7.56 (1H, d, $J = 8$ Hz, dichlorophenyl H_6), 7.68 (1H, dd, $J = 8.4$, $J' = 2$ Hz, dichlorophenyl H_5), 7.95 (1H, d, $J = 2$ Hz, dichlorophenyl H_2). Anal. calcd. for $C_{17}H_{19}Cl_2N_3O_4$: C, 51.01; H, 4.78; N, 10.50. Found: C, 50.88; H, 4.76; N, 10.67.

*Ethyl 1-{[5-(3,4-dichlorophenyl)-2-oxo-1,3,4-oxadiazol-3(2H)-yl]-methyl}piperidine-2-carboxylate (**5j**)*
White solid; yield: 59%; m.p. 80.7°C; IR (ν_{max}, KBr pellets, cm^{-1}): 3087–2860 (C–H), 1788 (C=O, RCOOR'), 1732 (C=O), 1614 (C=N), 1215, 1187 (C–O). ^{1}H NMR ($CDCl_3$) δ_H: 1.31 (3H, t, $J = 7.2$ Hz, $–CH_3$), 1.56–1.80 (6H, m, piperidine H_3, H_4, H_5), 2.66–2.72 (1H, m, piperidine H_6 axial), 3.19–3.24 (1H, m, piperidine H_6 equatorial), 3.45 (1H, q, piperidine H_2), 4.17–4.27 (2H, m, $COO–CH_2–$), 4.73 (1H, d, $J = 14.4$ Hz, methylene), 4.96 (1H, d, $J = 14$ Hz, methylene), 7.56 (1H, d, $J = 8.4$ Hz, dichlorophenyl H_6), 7.66 (1H, dd, $J = 8$, $J' = 2$ Hz, dichlorophenyl H_5), 7.93 (1H, d, $J = 2$ Hz, dichlorophenyl H_2). Anal. calcd. for $C_{17}H_{19}Cl_2N_3O_4$: C, 51.01; H, 4.78; N, 10.50. Found: C, 51.02; H, 4.92; N, 10.68.

Biological evaluation

Cell culture

RAW 264.7 macrophages were obtained from American Type Culture Collection (ATCC). The cells were cultured in DMEM, supplemented with 10% FBS and 1% streptomycin and penicillin at 37°C in 5% CO_2.

Cell cytotoxicity

RAW 264.7 cells at the density of 2×10^4 cells per well with 500 μL of culture medium were incubated for 24 h. The cells were treated with compounds for 24 h and then added 100 μL of 0.5 mg/mL MTT (AppliChem, Germany) for 2 h. The MTT solution was removed and 100 μL of isopropanol (Sigma–Aldrich, Germany) were added in each well and optical absorbance was measured at 570 nm.

Nitrite assay

RAW 264.7 cells were seeded into a 48-well culture plate at the density of 1×10^5 cells per well with 500 μL of culture medium and incubated for 24 h. The cells were then pretreated with compounds (**5a–j**) and reference molecule, indomethacin, at 100 μM for 2 h before stimulation with LPS (1 μg/mL) for 22 h. The nitrite concentration in the medium was measured by adding 50 μL Griess reagent [1% sulfanilamide (Sigma–Aldrich, USA) and 0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride (Sigma–Aldrich, USA) in 5% phosphoric acid (Mettler, Switzerland)] to 50 μL of medium for 10 min. The absorbance at 570 nm was then measured using a microplate reader (Microplate photometer, Multiskan Ascent, Finland). The amount of nitrite in the test samples was calculated from a sodium nitrite (Fluka Chemika, Germany) standard curve.

All results were expressed as the mean ± SD of experiments. Statistical significance was determined by one-way ANOVA followed by Tukey's test using a computerized statistical program. The data were considered statistically significant if $p < 0.05$.

In vivo anti-inflammatory activity assays

Animals

Sexually mature, 10- to 14-week-old male Sprague-Dawley rats (weighing 250–300 g) were obtained from Ondokuz Mayis University (Samsun, Turkey) vivarium sources. Animals were housed per group (n = 6, total 90 rats) in a quiet, temperature- and humidity-controlled room (22°C and 60 ± 5%, respectively) in a 12-h light/dark cycle, receiving food and water *ad libitum*. All procedures and protocols were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication 865-23, Bethesda, MD, USA).

Carrageenan footpad edema

The rats were deprived of food for 12 h prior to the beginning of experiments. Paw edema was induced by subplantar

injection of 100 μL of 1% sterile carrageenan lambda (Sigma, St. Louis, MO, USA) in saline into the right hind paw [24]. The edema component of inflammation was quantified by measuring the difference in hind footpad thickness before carrageenan injection and at 60, 120, 180, and 240 min after carrageenan injection with a micrometer caliper. Vehicle (0.5% carboxymethyl cellulose, 1 mL/rat) or drugs (100 mg/kg) were administered orally by gastric intubation to rats 30 min before carrageenan administration. Indomethacin (CAS 53-86-1) was used as a standard at the dose of 10 mg/kg by the same application route.

Gastric ulceration study

After anti-inflammatory activity study, animals were sacrificed by cervical dislocation. The stomach was removed and opened along the greater curvature. Active hemorrhagic foci were evaluated under the dissecting microscope.

Statistical analysis

All data are expressed as means ± SEM. All data analyses were performed using the GraphPad Instat (v3.0) software (GraphPad software, USA). Following the assurance of normal distribution of data, one-way analysis of variance (ANOVA) with the Tukey-Kramer *post hoc* test was used for multiple comparison. Values of $p < 0.05$ were regarded as significant.

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