Novel coumarin–benzimidazole derivatives as antioxidants and safer anti-inflammatory agents

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Abstract Inspired from occurrence of anti-inflammatory activity of 3-substituted coumarins and antiulcer activity of various 2-substituted benzimidazoles, novel compounds have been designed by coupling coumarin derivatives at 3-position directly or through amide linkage with benzimidazole nucleus at 2-position. The resultant compounds are expected to exhibit both anti-inflammatory and antioxidant activities along with less gastric toxicity profile. Two series of coumarin–benzimidazole derivatives (4a–e and 5a–e) were synthesized and evaluated for anti-inflammatory activity and antioxidant activity. Compounds 4c, 4d and 5a displayed good anti-inflammatory (45.45%, 46.75% and 42.85% inhibition, respectively, versus 54.54% inhibition by indomethacin) and antioxidant (IC50 of 19.7, 13.9 and 1.2 μmol/L, respectively, versus 23.4 μmol/L for butylated hydroxytoluene) activities. Evaluation of ulcer index and in vivo biochemical estimations for oxidative stress revealed that compounds 4d and 5a remain safe on gastric mucosa and did not induce oxidative stress in tissues. Calculation of various molecular properties suggests the compounds to be sufficiently bioavailable.

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1. Introduction

Inflammation is an important indication in many pathological conditions such as rheumatoid arthritis, osteoarthritis, gout, Alzheimer's disease and obesity related diseases. Chronic inflammatory states lead to a vicious cycle of inflammation and the accompanying pathological states, like obesity can lead to inflammation and the chronic inflammation can promote obesity associated diabetes by inducing insulin resistance. Therefore, control of inflammation becomes more important in all pathological conditions. The well-known non-steroidal anti-inflammatory drugs (NSAIDs) viz. indomethacin, ibuprofen and naproxen are commonly employed drugs in first line treatment of various chronic inflammatory disease states.

The major limitation of use of NSAIDs is gastric intolerance, which is manifested by dyspepsia, bleeding and ulcers. It occurs due to prostaglandin synthesis blockade as a result of cyclooxygenase (COX) inhibition by NSAIDs as well as due to acidic character of the NSAIDs themselves. Users of NSAIDs are found to be at 3 times greater risk of developing serious gastro-intestinal (GIT) adverse effects than the nonusers. Another statistical analysis displays that 23%–31% of patients develop gastric lesions when prescribed NSAIDs for arthritis. All these facts have aroused the need to improve the safety profile of existing NSAIDs or to discover better alternatives. Various COX-2 selective inhibitors have exhibited marked anti-inflammatory effect with reduced GIT toxicity. MK-0966, rofecoxib and celecoxib are selective COX-2 inhibitors with significant anti-inflammatory activity but induce less GIT side-effects in comparison to those of aspirin and ibuprofen.

Coupling of NSAIDs with an antioxidant cysteamine has produced compounds having good activity with less GIT intolerance. Coupling of nitric oxide, a cellular antioxidant with NSAIDs has also been explored successfully to design anti-inflammatory agents with markedly reduced ulcerogenic potential.

Coumarins form an elite class of compounds, which exhibit a variety of therapeutic activities including antioxidant, anti-inflammatory, antitumor, antiviral, antituberculosis and antimicrobial. Anti-inflammatory activity of coumarin derived compounds has been reviewed extensively and a structure activity relationship SAR has been established wherein it is found that an aromatic group when directly fused or linked through amide linkage at 3-position of coumarin nucleus incurs anti-inflammatory activity (Fig. 1). Many such derivatives also possess antioxidant activity through scavenging mechanisms.

Benzimidazole is another multifacet nucleus possessing a wide range of biological activities. This nucleus bearing at its 2-position a heterocycle through linker has been found in many clinically available antitumor drugs. It reveals that benzimidazole substituted with an appropriate group at 2-position is an important structural feature for gastric safety of the molecule. Therefore, the present study is undertaken to design novel molecules through coupling of 2-position of benzimidazole nucleus with 3-position of 6-substituted coumarin nucleus (Fig. 1), which can be exploited as viable alternatives to the existing NSAIDs. The resultant molecules are expected to exhibit both anti-inflammatory and antioxidant activities but still being less gastro-toxic.

2. Results and discussion

2.1. Chemistry

The target compounds were synthesized as shown in Scheme 1. Initially, Meldrum's acid (1) was prepared by treating malonic acid with acetone in the presence of catalytic amounts of sulphuric acid and acetic anhydride. Different salicylaldehydes (2a–e) were reacted with 1 in the presence of piperidinium acetate to obtain 6-substituted coumarin-3-carboxylic acids (3a–e). These intermediates were then used to synthesize target compounds 4a–e by refluxing them with o-phenylenediamine under inert environment (nitrogen) in the presence of catalytic amounts of polyphosphoric acid (PPA). The target compounds 5a–e were synthesized by coupling these intermediates with 2-aminobenzimidazole in the presence of dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopropylidine (DMAP). Many literature reports reveal the use of both orthophosphoric acid (OPA) and PPA for the formation of benzimidazole nucleus. In the present study, PPA was used as catalyst as OPA produced more byproducts and took longer reaction times. All target compounds were obtained in good yields and were found to be pure as assured by single spots in thin layer chromatographic plates (TLC). Structures of the synthesized compounds were confirmed by IR, NMR and high resolution mass (HRMS) spectral techniques. Formation of benzimidazole nucleus in compounds 4 and 5 was ascertained by the disappearance of COOH band, due to COOH group of compounds 2, in their IR spectra. In addition, IR spectra of 5 showed an amide band in the range of 1640–1690 cm$^{-1}$. Presence of benzimidazole nucleus was confirmed by appearance of signals due to –NH and four aromatic protons of the nucleus in their $^1$H NMR spectra. The labile –NH protons were detected in the range of 6.89–9.2, which was confirmed by deuteration exchange experiments. The $^{13}$C NMR spectra showed distinct resonances in agreement with the proposed structure. The methoxy derivatives 4b and 5b showed distinct peaks due to methoxyl carbon at around 65. The benzimidazole carbons were detected at $\delta$ 116.21–125.51, and the coumarinyl carbonyl carbon was found at $\delta$ 155.62–164.05. Carbon atoms of the benzene ring of coumarin nucleus showed downfield or upfield shifts in consonant with the type of substituent present on the ring. Finally, the HRMS data, recorded with electrospray ionization in positive polarity (+ESI), of each compound showed that the mass of [M+H$^+$ ion was in close agreement with its accurate theoretical mass.

2.2. Anti-inflammatory activity

It was evaluated in terms of percent (%) inhibition of formalin induced oedema in rat paw. The activity was monitored at 0.5, 1, 2, 4 and 6 h after administration. It was found that the activity continuously increased with time. All test compounds exhibited good to moderate anti-inflammatory activity, which was comparable to indomethacin at each time period (Fig. 2). Further, the inhibition profile of each compound was similar to that of indomethacin at each time slab, which suggested that the mechanism of action of the compounds might be similar to that of indomethacin. Compounds 4a–e showed anti-inflammatory effects better than those of compounds 5a–e. Compounds 4d and 4c were maximally potent with 46.75% and 45.45% inhibition of paw oedema, respectively. From the other series, compound 5a was the most potent with 42.85% inhibition. These results suggest that an electron withdrawing group (–Cl or –Br) increases the anti-inflammatory potency whereas electron releasing group (–OCH$_3$) decreases the potency. Further, it was found that an amide linkage in the molecule decreased the activity.

2.3. In vitro antioxidant activity

The antioxidant potential was evaluated as radical scavenging capacity using 2,2-diphenyl-1-picrylhydrazyl (DPPH) method. DPPH being
composed of unstable free radical traps any radicals produced by the test compound and bring about a visible colour change ranging from violet to pale yellow to colourless. The degree of change in absorbance with respect to control is calculated as antioxidant potential. Due to the non-linear relationship in the DPPH method, probit regression was applied and EC50 values were calculated using BLeSq software.\(^2\) Series 5a–e as depicted in Table 1. Compounds 4d and 5a were found to be most potent with EC50 value \(13.92 \pm 1.4 \mu\text{mol/L}\) and \(1.2 \pm 0.1 \mu\text{mol/L}\), respectively. The study reveals that electron withdrawing groups increase the antioxidant potential which may be due to the intensification of positive charge on –NH of amide associated with negative inductive effect of these groups. The positive charge intensification may lead to free radical quenching. Moreover, electron withdrawing groups are themselves good free radical quenchers. On the contrary, substitution with electron releasing groups was found to decrease the radical scavenging potential possibly due to their positive inductive effect.

2.4. Gastric safety and in vivo oxidative stress

Based on anti-inflammatory and in vitro antioxidant activities, the compounds 4c, 4d and 5a were selected for evaluation of gastric safety as well as in vivo oxidative stress. The compounds 4d and
5a were found to be safe on gastric mucosa as indicated by their low ulcer index (0.67 and 0.75, respectively) in comparison to indomethacin which has scored an ulcer index of 3.17 (Table 2). The increased catalase and glutathione levels and decreased thiobarbituric acid reactive substances (TBARS) levels in animals treated with standard drug with respect to the control group indicated that indomethacin had induced oxidative stress in the tissues. In consonant with the gastric safety profile, the compounds 4d and 5a have been found to exert no oxidative stress on the tissue as indicated by the catalase, glutathione and TBARS levels being almost equal to those of control group.

These results indicated the compounds 4d and 5a to be maximally safe on gastric mucosa as well as inducing negligible oxidative stress. The lower gastric safety and relatively poor oxidative stress parameters of compound 4c may be attributed to the presence of bromo group in the molecule.

2.5. Molecular properties calculation

“Lipinski’s rule of 5” predicts oral bioavailability, intestinal absorption and blood brain barrier permeability of a new molecule through its molecular properties that include lipophilicity (log P), total polar surface area (TPSA), nON (number of H-bond acceptors), nOHNH (number of H-bond donors) and molecular weight. These properties are calculated using molinspiration calculations software. Log P and TPSA are the two most important properties for this prediction. TPSA is closely related to hydrogen bonding potential of a compound. Molecules with TPSA of about 160 Å or more are expected to have poor intestinal absorption.

All compounds have been found to have nON and nOHNH in the ranges of 4–9 and 1–2, respectively, molecular weights less than 400, Log P in the range of 2.5–4.3, and TPSA in the range of 58–133. Moreover, all the compounds have zero violation of this rule. Hence, these parameters suggest that the compounds are expected to exhibit good oral bioavailability and intestinal absorption.

3. Conclusions

Two series of compounds were designed and synthesized by conjugating coumarin at 3-position with benzimidazole at 2-position through a single bond (series 4) as well as an amide linkage (series 5). The series 4 was found to possess anti-inflammatory activity better than the series 5. Compounds 4c, 4d and 5a exhibited maximum inhibition of inflammation. On the contrary, series 5 exhibited very potent

**Table 1** *In vitro* antioxidant activity by DPPH method.

| Compound | Inhibition (%) | EC_{50} (µmol/L) |
|----------|---------------|-----------------|
|          | 1 µmol/L | 2 µmol/L | 5 µmol/L | 10 µmol/L | 20 µmol/L | 50 µmol/L |          |
| 4a       | 2.2 ± 0.1  | 7.5 ± 0.4  | 25.2 ± 1.6 | 47.1 ± 3.3 | 62.8 ± 6.4 | 69.5 ± 4.4 | 15.5 ± 1.5 |
| 4b       | 15.1 ± 0.5 | 17.2 ± 0.6 | 22.9 ± 1.2 | 25.5 ± 1.8 | 28.7 ± 1.3 | 35.3 ± 1.5 | > 50.0   |
| 4c       | 1.6 ± 0.1  | 6.1 ± 0.2  | 19.8 ± 1.8 | 37.8 ± 3.1 | 56.2 ± 3.0 | 65.4 ± 4.3 | 19.7 ± 1.7 |
| 4d       | 0.7 ± 0.1  | 4.1 ± 0.3  | 22.0 ± 0.8 | 32.6 ± 2.7 | 63.8 ± 3.6 | 70.1 ± 4.3 | 13.92 ± 1.4 |
| 4e       | 0.4 ± 0.01 | 3.8 ± 0.2  | 18.8 ± 1.8 | 37.2 ± 2.5 | 50.9 ± 4.7 | 59.5 ± 3.0 | 22.5 ± 1.7 |
| 5a       | 45.3 ± 3.8 | 55.8 ± 2.2 | 64.1 ± 4.0 | 69.0 ± 4.8 | 71.6 ± 2.8 | 75.8 ± 3.7 | 1.2 ± 0.1 |
| 5b       | 0.7 ± 0.04 | 2.7 ± 0.1  | 22.19 ± 0.4 | 30.0 ± 4.0 | 39.0 ± 1.9 | 56.3 ± 3.9 | 28.5 ± 1.8 |
| 5c       | 36.0 ± 0.9 | 55.8 ± 3.0 | 64.5 ± 2.4 | 68.0 ± 2.5 | 71.1 ± 4.9 | 75.9 ± 4.4 | 1.9 ± 0.1 |
| 5d       | 17.2 ± 0.6 | 41.3 ± 3.0 | 60.1 ± 3.6 | 71.8 ± 3.4 | 76.3 ± 5.4 | 90.5 ± 4.9 | 4.0 ± 0.4 |
| 5e       | 25.4 ± 0.6 | 42.7 ± 1.5 | 55.0 ± 2.3 | 59.7 ± 3.8 | 64.1 ± 3.4 | 75.9 ± 4.9 | 5.0 ± 0.5 |
| BHTb     | 5.6 ± 0.7  | 19.2 ± 1.8 | 28.0 ± 1.7 | 37.5 ± 2.8 | 48.9 ± 3.7 | 60.4 ± 2.9 | 23.4 ± 3.1 |

*EC_{50}: concentration which possesses 50% radical scavenging ability.

*Butylatedhydroxytoluene.

**Figure 2** Anti-inflammatory effect of compounds of series 4 and 5. Values are represented as mean ± SD (n=6). *Values are statistically different from standard at each time interval, P<0.05.
antioxidant activities with 5a being the most potent and even more active than the standard compound BHT. SAR was established on the basis of the results obtained. Maximally potent compounds were found to be safe on gastric mucosa with least ability to induce oxidative stress in tissues except compound 4c. All compounds possessed sufficiently good oral bioavailability and hence, compounds 4d and 5a could be taken as lead for development of potent anti-inflammatory and antioxidant activities yet being safe to gastric mucosa.

4. Experimental

The reagents and solvents were of laboratory grade and were procured from different suppliers (LobaChemie, Mumbai; SD Fine, Mumbai; Merck, Mumbai). Melting points were determined in open capillaries using Digital Auto Melting Point Apparatus (Labtronics) and are uncorrected. Purity of the compounds was ascertained by TLC using precoated aluminium TLC plates visualized in a UV/Iodine chamber. Infrared spectra were recorded on an Alpha-E FTIR spectrophotometer (BrukerOptik, Germany) using potassium bromide optics. 1H and 13C NMR spectra were recorded on a BrukerAvance II spectrometer (400 MHz) using tetramethylsilane (TMS) as internal standard and chemical shifts are given in ppm. Mass spectra were recorded using a Q-TOF micromass spectrometer (Waters, MA, USA).

Wistar rats (150–250 g) of either sex were employed for the study. They were exposed to 12 h light/dark cycle and the animals had free access to food and water. The animals were given standard laboratory pellet chow diet and water ad libitum, both being withdrawn 12 h prior to experiment. The experimental protocol was duly approved by Institutional Animal Ethical Committee and the care of animals was done as per guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals, Government of India. The animals were administered orally with 20 mg/kg of indomethacin and the test compounds at equimolar doses to standard drug.

4.1. Chemistry

Synthesis of Meldrum’s acid (1) and intermediate compound 3a (2-oxo-2H-chromen-3-carboxylic acid) has been reported in literature27,28. Other derivatives of series 3 are novel compounds whose spectral data are as follows:

6-Methoxy-2-oxo-2H-chromen-3-carboxylic acid (3B): Yield: 88%; pale yellow crystals; mp: 210–212 °C; 1H NMR: δ 11.32 (s, 1H), 8.69 (s, 1H), 7.39 (d, 1H, J=2.88 Hz), 7.32–7.34 (m, 1H), 7.28 (dd, 1H, J_A=2.88 Hz, J_B=6.2 Hz), 3.83 (s, 3H); 13C NMR: δ 162.78, 158.53, 155.62, 148.09, 124.26, 122.35, 119.02, 118.81, 118.06, 111.67, 55.83; IR (KBr): 3048, 2990, 2763–3048, 1758, 1610, 1490, 1460, 1380, 1250, 1200, 1150, 1090, 1030, 920, 840, 750, 690, 620 cm⁻¹. 

Table 2  Biochemical estimations and ulcer index of 4c, 4d and 5a.

| Compound | Catalase (µmol/L/mg) | TBARS (nmol/L/mg) | Glutathione (µmol/L/100 mg) | Ulcer index |
|----------|----------------------|------------------|-----------------------------|-------------|
| Control  | 22.14 ± 0.98         | 0.63 ± 0.09      | 184.10 ± 5.03              | 0.17 ± 0.25 |
| Standard (ID) | 7.79 ± 0.19     | 5.32 ± 0.11      | 85.20 ± 3.10               | 3.17 ± 1.03 |
| 4c       | 15.83 ± 3.21         | 1.67 ± 0.15      | 87.09 ± 4.04               | 2.25 ± 0.31 |
| 4d       | 20.46 ± 2.02         | 0.98 ± 0.02      | 169.84 ± 5.09              | 0.67 ± 0.25 |
| 5a       | 21.96 ± 1.70         | 0.89 ± 0.07      | 176.41 ± 3.19              | 0.75 ± 0.25 |

aValues are statistically different from control, P<0.05.
bValues are statistically different from standard drug, P<0.05.

Table 3  TPSA and molecular properties of test compounds.

| Compound | TPSA a | Log P b | MW c | nON d | nOHNH e | n viol f | n rotbg |
|----------|--------|---------|------|-------|---------|----------|---------|
| 4a       | 58.894 | 3.549   | 262.268 | 4     | 1       | 0        | 1       |
| 4b       | 68.128 | 3.582   | 292.294 | 5     | 1       | 0        | 2       |
| 4c       | 58.894 | 4.334   | 341.164 | 4     | 1       | 0        | 1       |
| 4d       | 58.894 | 4.203   | 296.713 | 4     | 1       | 0        | 1       |
| 4e       | 104.718| 3.484   | 307.265 | 7     | 1       | 0        | 2       |
| 5a       | 87.992 | 2.645   | 305.293 | 6     | 2       | 0        | 2       |
| 5b       | 97.226 | 2.677   | 335.319 | 7     | 2       | 0        | 3       |
| 5c       | 87.992 | 3.43    | 384.189 | 6     | 2       | 0        | 2       |
| 5d       | 87.992 | 3.299   | 339.738 | 6     | 2       | 0        | 2       |
| 5e       | 133.816| 2.58    | 350.29  | 9     | 2       | 0        | 3       |

aTotal polar surface area.
bLipophilicity.
cMolecular weight.
dHydrogen bond acceptors.
eHydrogen bond donors.
fNumber of violations.
gNumber of rotatable bonds.
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1671, 1494, 1405, 1237 cm⁻¹; HRMS (ESI+) m/z: calc. for C₁₇H₁₂N₃O₂: 263.0815, found 263.0824.

3-(1H-Benz[d]imidazol-2-yl)-6-nitro-2H-chromen-2-one (4e): Yield: 60%; green amorphous solid; mp > 290 °C; 1H NMR: δ 9.29 (s, 1H), 8.50 (d, 2H, J = 9.76 Hz), 7.75–7.85 (m, 2H), 7.54–7.66 (m, 2H), 7.31–7.32 (m, 2H); 13C NMR: δ 162.92, 155.36, 146.68, 144.61, 143.77, 141.12, 140.36, 128.98, 14.58, 132.24, 122.82, 122.55, 118.28, 11.18, 116.32; IR (KBr): 3119, 1750, 1646, 1546, 1497, 1330, 1220 cm⁻¹; HR-MS (ESI+) m/z: calc. for C₁₇H₁₀ClN₃O₂: 397.0425, found 397.1436.

4.1.2. Synthesis of test compounds 5a–e
A suspension of 3a–e (0.01 mol) and DCC (2.3 g, 0.011 mol) in dried dichloromethane (DCM) (100 mL) was vigorously stirred for 30 min under nitrogen. A solution of 2-aminobenzimidazole (0.1 mol) dissolved in dried DCM (30 mL) and freshly distilled pyridine (50 mL) along with DMAP (0.050 g) was added to the reaction mixture at 0 °C in 15 min. The solution was stirred at 0 °C for 2 h followed by overnight stirring at room temperature. The solution was filtered to remove diclohexylurea and the filtrate was evaporated in vacuum to yield dry solid which was dissolved in dry ethyl acetate with heating in a water bath. The residue was filtered and the filtrate was washed with distilled water. The ethyl acetate layer was dried with magnesium sulphate and evaporated in vacuum. The resulting crude solid was recrystallized from methanol to yield corresponding amide. The spectral data of the compounds of series 5a–e as follows:

N-(1H-Benz[d]imidazol-2-yl)-2-oxo-2H-chromen-3-carboxamide (5a): Yield: 54%; yellow amorphous solid; mp: 198–200 °C; 1H NMR: δ 9.14 (s, 1H, NH), 8.30 (s, 1H, J = 1.44 Hz, J = 6.32 Hz), 7.31–7.38 (m, 2H), 7.24–7.26 (m, 2H), 6.45 (br s, 1H); 13C NMR: δ 163.21, 156.58, 153.25, 152.81, 143.57, 142.16, 132.97, 130.59, 128.87, 124.57, 124.57, 122.93, 118.39, 117.29, 116.21, 116.21, 114.81; IR (KBr): 3054, 1707, 1664, 1644, 1531, 1315, 1225 cm⁻¹; HR-MS (ESI+) m/z: calc. for C₁₇H₁₀N₄O₂: 308.0666, found 308.0615.
7.40 (m, 2H), 5.57 (br s, 1H); 13C NMR: δ 162.96, 159.86, 152.78, 151.75, 137.24, 135.81, 134.61, 132.76, 125.51, 124.68, 1708, 1690, 1645, 1534, 1266, 1044 cm⁻¹; IR (KBr): 3050, 1738, 1709, 1680, 1561, 1458, 1402, 1282, 1243, 1221, 119.01, 117.12, 116.81, 114.97; HR-MS: calcd. for C₁₇H₁₁ClN₃O₂: 340.0483, found 340.0498.

N-(1H-Benzol[d]imidazol-2-yl)-6-nitro-2-oxo-2H-chromen-3-carboxamide (5e): Yield: 56%; yellow amorphous solid; mp ≥ 290 °C; 1H NMR: δ 9.15 (s, 1H), 8.84 (d, 1H, J = 2.72 Hz), 8.41–8.52 (m, 1H), 8.31–8.34 (m, 1H), 7.61–7.70 (m, 1H), 7.40–7.45 (m, 1H), 7.33–7.35 (m, 1H), 7.20–7.22 (m, 2H), 6.89 (br s, 1H); 13C NMR: δ 163.29, 159.48, 158.65, 151.21, 145.89, 140.27, 139.94, 124.45, 124.45, 124.24, 123.79, 123.50, 122.11, 119.01, 117.12, 116.81, 114.97; IR (KBr): 3050, 1738, 1709, 1680, 1561, 1529, 1268, 1229 cm⁻¹; HR-MS (+ESI) m/z: calcd. for C₁₇H₁₂N₃O₃: 351.0724, found 351.0740.

4.2. Anti-inflammatory activity

The inflammation was induced by injecting 0.1 mL of formalin in subplantular region of rat’s hind paw. Animals were divided into various groups each of six rats. The control group received vehicle (0.5% sodium carboxymethylcellulose (SCMC)) whereas the standard group received the standard drug indomethacin at the therapeutic dose for anti-inflammatory activity. The test compounds at the dose equimolar to the standard drug 1 h prior to the formalin injection. The paw volume was measured by plethysmograph and the change in paw volume was noted periodically over 0–6 h.° inhibition was calculated by the formula reported by Chu and Kovacs.°

Inhibition(%) = 100 – [(oedema volume in treated/oedema volume in control) × 100]

4.3. Antioxidant activity

4.3.1. DPPH assay

The antioxidant activity of the test compounds was evaluated in terms of hydrogen donating or radical scavenging ability with the DPPH method taking BHT as standard drug.° The activity was evaluated by taking different concentrations of each test and standard compound. 700 µL of each concentration of the standard solution of ascorbic acid and test compounds in methanol was mixed with the same volume of methanolic solution of 700 µm/L DPPH. The mixed solution was shaken vigorously, allowed to stand in dark at room temperature for 30 min and its absorbance was read at 515 nm using a UV–Vis spectrophotometer (Beckman, USA). The standard/test solution was replaced with methanol to serve as control. The antiradical activity was calculated in terms of inhibition using the following equation: ([AbsControl − AbsTest]/AbsControl) × 100%. Different sample concentrations of each test compound and standard were used in order to obtain a calibration curve for each test compound as well as BHT was constructed by taking inhibition(%) as abscissa and concentration as coordinate to calculate the EC₅₀ values (the concentration required to obtain a 50% radical scavenging activity). Data analysis was performed as reported by Locatelli et al.° to compute EC₅₀ on the basis of probit regression. The antioxidant activity of each test compound was performed in triplicate and EC₅₀ values are reported as mean ± standard deviation (SD).

4.3.2. In vivo biochemical estimations

Glandular parts of the extracted stomachs were homogenized in cold phosphate buffer (pH 7.4) for 2 min. The homogenized contents were centrifuged at 800 g for 20 min followed by centrifugation at 12,000 g for 15 min. The resulting supernatant was used for catalase,° lipid peroxidation (LPO)° and glutathione reductase assays.

4.4. Chronic ulcerogenicity test

Albino rats were used to perform this test. The control group received vehicle whereas the test group and the standard group received the test compounds (4 and 5) respectively and the standard drug (indomethacin) at the therapeutic dose for anti-inflammatory effects for a period of 28 days orally. The rats were then sacrificed and the stomach was removed and opened along the greater curvature. The inner surface was washed slowly with normal saline and was examined for the severity of ulceration according to the following scale: 0 = normal grey coloured stomach, 0.5 = pink to red colouration of stomach, 1 = spot ulcer, 1.5 = haemorrhagic ulcer, 2 = ulcer < 5, 3 = ulcer > 5, 4 = ulcers with bleeding. Mean ulcer score for the control, standard and each test compound was calculated and reported as ulcer index.°

4.5. Statistical analysis

All results were expressed as mean ± SD. The statistical significance was determined by one-way analysis of variance (ANOVA) followed by Dunnet’s test and the results were found significant at P < 0.05.

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