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Identification and evaluation of antioxidant, analgesic/anti-inflammatory activity of the most active ninhydrin–phenol adducts synthesized

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Abstract—Treatment of phenols with ninhydrin in acidic medium afforded 2-hydroxy-2-(*ortho*-hydroxy-phenyl/naphthyl)-1,3-dioxo-indanes, which being unstable were isolated in their hemiketal forms. These synthesized compounds were subjected to TLC screening for radical scavenging and in vitro lipoxgenase and cycloxygenase enzyme inhibition assays. The best compound was identified and studied in detail for steady-state and time-resolved free radical kinetics, viz., DPPH, $ABTS^{\cdot-}$, $^{\cdot}OH$ and rate constants for these reactions were evaluated. The best compound was also subjected to in vivo anti-inflammatory and analgesic activities in which the compound showed good promise for further structural optimization.

1. Introduction

A serendipitous observation while identifying one of the plant constituents using ninhydrin as chromogenic reagent resulted in a compound with potential free radical scavenging properties (Data unpublished). This led us to synthesize and screen alternate analogues with ninhydrin as one of the substrate. It is reported that the C-2 position of the ninhydrin is more reactive towards nitrogen, sulfur, oxygen and carbon based nucleophiles.[1,2] The acetic acid catalyzed reaction of ninhydrin with amino, C-alkyl, hydroxy, and alkoxy phenols has been studied and their mechanism of action has also been postulated.[3] The acid catalyzed reaction of ninhydrin with various aromatic and non-aromatic substrates using mild and micro oven reaction enhanced (MORE) conditions has been reported.[4] We thought of exploring and screening the monoarylated adducts formed by the reaction of ninhydrin with polyhydroxy benzenes and α-naphthol toward free radical scavenging activity. The most promising molecule from the set of synthesized compounds was identified using in situ DPPH scavenging on TLC. The structures of the compounds were also screened on-line using PASS (prediction of activity spectra for substances) software.[5,6] Further, the best compound was screened for detailed free radical scavenging ability in steady-state and kinetic conditions; in vitro lipoxgenase and cycloxygenase inhibition studies; in vivo anti-inflammatory and analgesic activities.

2. Results and discussion

2.1. Chemistry

Ninhydrin was made to react with phenols, viz., catechol (**2a**), resorcinol (**2b**), orcinol (**2c**), pyrogallol (**2d**), and α-naphthol (**2e**) in acetic acid medium at 40 °C by stirring on a magnetic stirrer for varying periods from 15 to 30 min to yield monoarylated adducts (Scheme 1). All the adducts formed (**4a**–**e**) were recrystallized by using appropriate solvents and DSC, IR, ^{1}H NMR spectra were recorded, and the peaks were matched with those reported in the literature.[4] The 2-hydroxy-2-(*ortho*-

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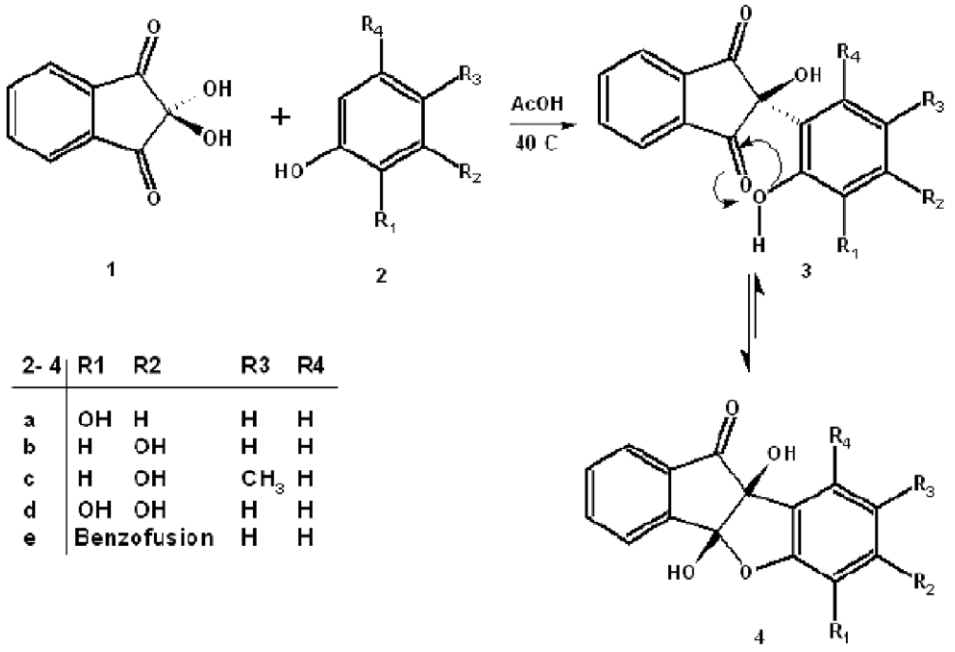


2-4	R1	R2	R3	R4
a	OH	H	H	H
b	H	OH	H	H
c	H	OH	CH_3	H
d	OH	OH	H	H
e	Benzofusion		H	H

Scheme 1.

hydroxy-phenyl/naphthyl)-1,3-dioxoindanes (**3**) formed are reported to be in stable intramolecular hemi-ketal form **4**, which is also supported by the reported X-ray studies.[4]

2.2. TLC screening for free radical scavengers

All the synthesized compounds (**4a**–**e**) along with ninhydrin (**1**) were developed on a TLC plate and sprayed with DPPH solution, air-dried, and observed visually for scavenging of DPPH. The rapid scavenging of DPPH was observed with the compound **4e** in just few seconds compared to all others.

2.3. 5-Lipoxygenase (5-LOX) and cycloxygenase (COX) inhibition

The 5-LOX inhibitory activities of **4a**–**e** were determined using enzyme isolated from potato and by the method of Shimizu et al. The compound **4e** alone was found to possess an IC_{50} of 17.2 μM toward inhibition of 5-LOX but none of the other tested compounds show any inhibitory activity. Lipoxygenases (LOXs) constitute a heterogeneous family of lipid peroxidizing enzymes, capable of oxygenating polyunsaturated fatty acids to their corresponding hydroperoxy derivatives. Leukotrienes (LTs) are potent mediators of inflammation derived from arachidonic acid through the action of 5-LOX.[7] Several peptidoleukotrienes are powerful spasmogens and are implicated in inflammation and several allergic processes.[8] Thus, inhibition of leukotriene synthesis through inhibition of 5-LOX is considered as one of the new potential targets for the treatment of asthma and rheumatoid arthritis.[9] Inhibitors of 5-LOX are classified as redox inhibitors, iron-ligand inhibitors, and non-redox inhibitors.[10] Compound **4e** shows very good free radical scavenging properties, thus it may probably act as redox inhibitor of 5-LOX and could be a good hit for further studies as 5-LOX inhibitors.

The COX1 and COX2 inhibitory activities were studied by using the method described by Reddanna et al. None of the compounds screened, however, showed any inhibitory activity.

Based on the results of TLC screening and 5-LOX inhibition studies, **4e**, which showed maximum activity, was chosen as a potential candidate for further investigations such as free radical scavenging and in vivo analgesic and anti-inflammatory studies.

2.4. Free radical reactions

2.4.1. Reaction with stable DPPH radical. The Scavenging ability of **4e** toward the stable DPPH (50 μM) free radical was studied and found to scavenge with an IC_{50} of 32.82 μM. The scavenging ability of standard ascorbic acid under the same experimental conditions was found to be 15.74 μM, suggesting **4e** to possess one half-hydrogen donor capacity as that of ascorbic acid (Fig. 1).

The rate of decay of DPPH radical by various concentrations of **4e** with time (100 s) was determined by stopped-flow method and the decay trace was fitted to an exponential function using an in-built software and resulting first order rate constant was measured ($k_{(obs)}$). This $k_{(obs)}$ was then plotted against various concentrations of **4e** (Inset Fig. 1) and from the slope, the bimolecular rate constant was determined to be $38.2 \pm 1.3\ M^{-1}\ s^{-1}$. The bimolecular rate constant of ascorbic acid under same experimental conditions was found to be $140 \pm 10\ M^{-1}\ s^{-1}$.

2.4.2. Reaction with $ABTS^{\bullet-}$. The electron transfer capability of **4e** was studied by using $ABTS^{\bullet-}$ decay, as measured by the decrease in absorbance at 635 nm. Compound **4e** was found to possess an IC_{50} of

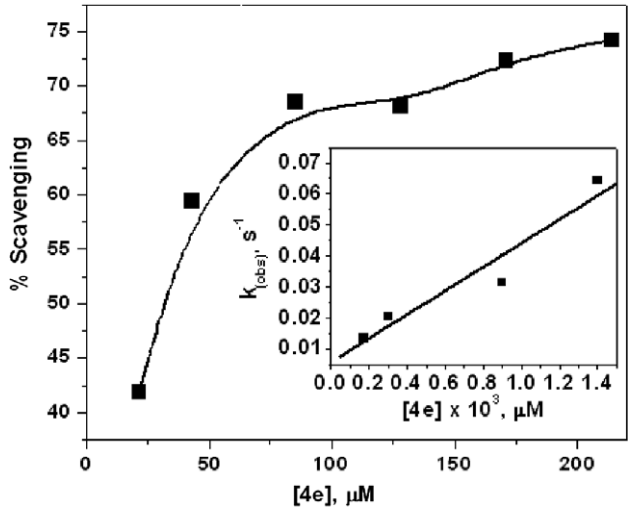


Figure 1. Percentage DPPH (50 μM) scavenging by different concentrations of **4e**; Inset: Linear plot of observed bimolecular rate constant against different concentrations of **4e**.

11.7 μM, which was comparable to ascorbic acid IC_{50} of 8.07 μM (Fig. 2).

The kinetics of the reaction between equivmolar concentrations of $ABTS^{\bullet-}$ and **4e** in a timescale of 10 s was studied using stopped-flow technique as described above. The bimolecular rate constant for this reaction was calculated by using ($k_{(obs)}$) obtained by fitting the decay trace to second order kinetics (as the concentrations of **4e** and $ABTS^{\bullet-}$ are similar), repeating the experiment with different concentrations and using an extinction coefficient of $1.35\times10^4\ M^{-1}\ cm^{-1}$ for $ABTS^{\bullet-}$ at 635 nm. From this the rate constant was found to be $3.0 \pm 0.8 \times 10^4\ M^{-1}\ s^{-1}$. A specimen decay curve of the reaction is shown as inset of Figure 2.

2.4.3. Reaction with $^{\bullet}OH$. The ability of **4e** to scavenge $^{\bullet}OH$ generated through Fenton reaction was estimated by the degradation of Deoxy-D-ribose yielding MDA, measured as TBARS. The compound **4e** possessed an IC_{50} of 31.12 μM in scavenging these $^{\bullet}OH$ (Fig. 3A). Mannitol was used as a standard in this assay.

The reaction of **4e** with the $^{\bullet}OH$ generated by using pulse radiolysis with an absorbed dose of 19.5 Gy was also studied. The transient spectrum obtained after pulse radiolysing N_2O saturated aqueous solution of 150 μM **4e** is given in Figure 3B, a. This spectrum shows one sharp peak at 330 nm and a broad featureless absorption at 400 nm region. Since $^{\bullet}OH$ reactions are non-specific, direct one-electron oxidation of **4e** is studied with sulfate ($SO_4^{\bullet-}$) radical and the transient spectrum of the reaction of **4e** (200 μM) with $SO_4^{\bullet-}$ is given in Figure 3B, b. It can be seen that the two spectra are significantly different indicating that $^{\bullet}OH$ does not react by oxidation and reacts probably by addition to one of the aromatic rings and this reaction is represented in Scheme 2. The bimolecular rate constant for this reaction was determined by following the rate of formation

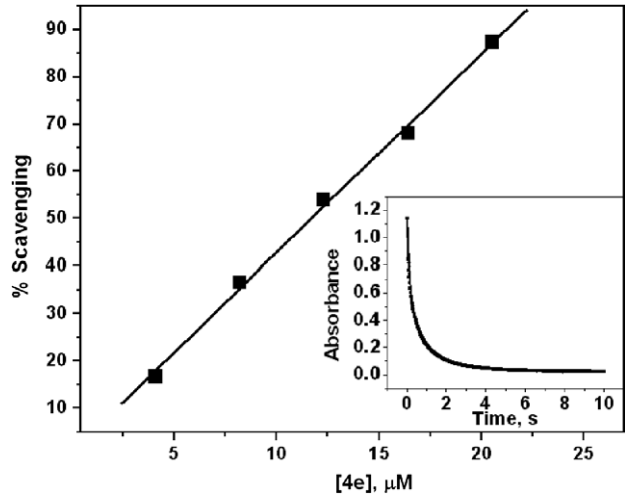


Figure 2. Percentage $ABTS^{\bullet-}$ (100 μM) scavenging by different concentrations of **4e**; Inset: Absorption-time plot showing the decay of $ABTS^{\bullet-}$ at 635 nm in presence of 100 μM **4e**.

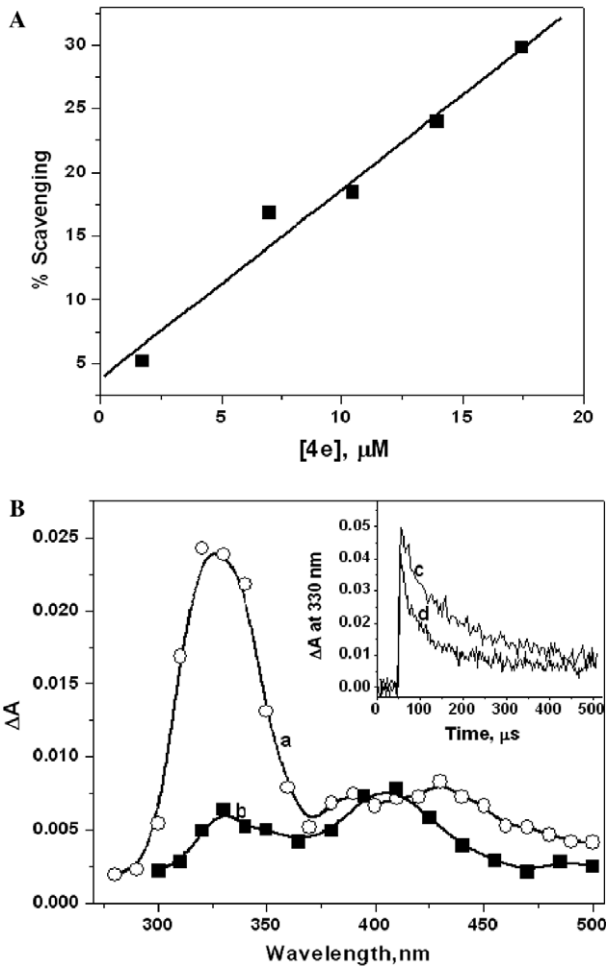
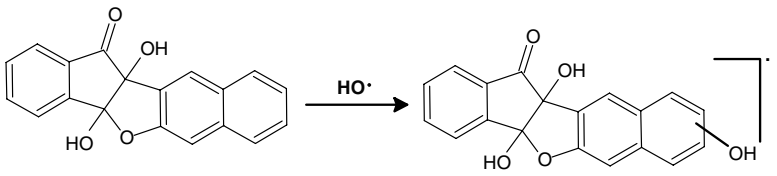


Figure 3. (A) $^{\bullet}OH$ (generated by Fenton reaction) scavenging by different concentrations of **4e** as assessed by deoxyribose degradation; (B) difference absorption spectra of the transient produced by the reaction of (a) $^{\bullet}OH$ with **4e** and $SO_4^{\bullet-}$ with **4e** at pH 7. Inset: absorption-time plots showing the decay of the transient produced by the reaction of $^{\bullet}OH$ with **4e** at 330 nm (c) in N_2O-saturated solutions and (d) in N_2O- and oxygen (80:20)-saturated solutions.



Scheme 2.

of the transient at 330 nm as a function of concentration (40–150 μM) and found to be 1.7×10^8 M^{-1} s^{-1}. The transient ·OH reaction decays by kinetics with a rate constant of 5.4×10^3 s^{-1}. In presence of oxygen it decays with a rate constant of $<1.9 \times 10^4$ s^{-1}, indicating a radical adduct formation with ·OH.

Reactive oxygen species (ROS), which includes superoxide anion, hydrogen peroxide, and ·OH, mediate cell damage in a variety of pathophysiological conditions and are responsible for oxidative injury of enzymes, lipid membranes, and DNA in living cells and tissues.[11] During the inflammatory process, phagocytes generate the superoxide anion radical at the inflammed site, which in turn leads to the formation of highly reactive ·OH which further causes lipid peroxidation; prostaglandins linked with such lipid peroxidation may amplify the inflammatory response. Compound **4e** with reasonably good rate constant for reaction with ·OH is expected to protect cells from oxidative damage.

2.4.4. In vitro lipid peroxidation assay. The ability of **4e** to inhibit iron-induced lipid peroxidation was studied using phosphatidylcholine liposomes with Trolox as a reference standard. Compound **4e** was found to possess an IC_{50} of 2.6 mM, while that of Trolox was found to be 18.6 μM (Fig. 4). As discussed above, lipid peroxidation is one of the important contributing factors for inflammatory response. Lipid peroxidation is also reported to increase phospholipase A_2 activity, which further contributes to cell injury.[12] Compound **4e** is found to inhibit lipid peroxidation thereby probably contributing to its overall anti-inflammatory activity.

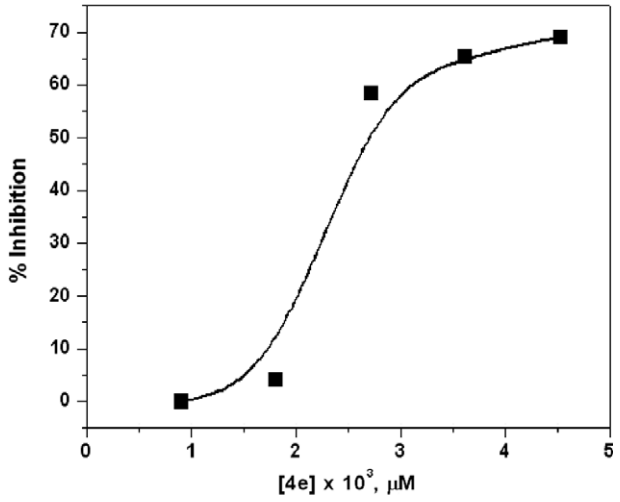


Figure 4. Inhibition of lipid peroxidation in egg phosphatidylcholine liposomes by different concentrations of **4e**.

2.5. Pharmacology

2.5.1. Acute toxicity studies. Compound **4e** did not produce any death at 1200 mg/kg bw ip whereas 50% death occurred within 24 h at 1800 mg/kg bw ip. No animal survived beyond 24 h at a dose of 2000 mg/kg bw. In all dose groups, the animals, which did not die within 72 h, survived up to 30 days without any apparent adverse symptoms. The LD_{50} for 30 days was calculated to be 1621.8 mg/kg bw.

2.5.2. Analgesic and anti-inflammatory studies. The effect of **4e** and Diclofenac as standard on Carrageenan-induced edema at different time intervals is depicted in Figure 5A. Acute inflammation is due to the release of chemical mediators, which cause edema as a result of extravasations of fluid and proteins from the local microvasculature and accumulation of polymorphonuclear leukocytes at the inflammatory site. Carrageenan-induced inflammation is a non-specific inflammation resulting from a complex of diverse mediators.[13] This model is conventional, sensitive, and accepted for screening of newer anti-inflammatory agents.[14] Further, this model reliably predicts the anti-inflammatory effica-

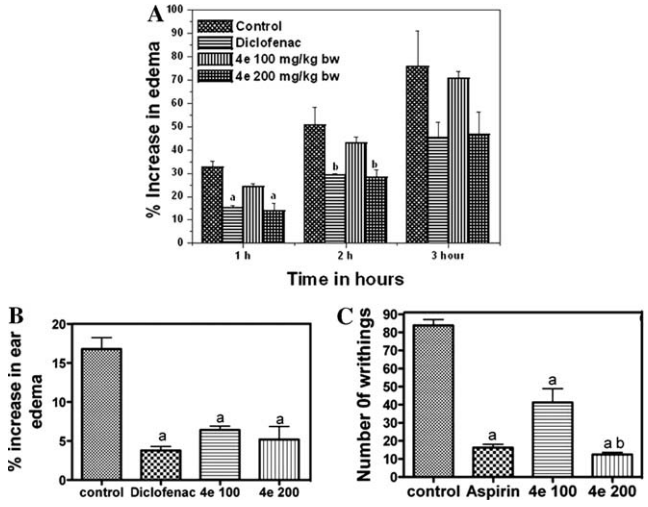


Figure 5. Analgesic and anti-inflammatory studies of **4e** (A). Effect of different concentrations of **4e** in carrageenan-induced rat paw edema measured at different time intervals, $^a p < 0.05$; $^b p < 0.01$ compared to control, $n = 5$; (B) effect of different concentrations of **4e** in oxazolone induced ear edema in mice; $^a p < 0.001$ compared to control, $n = 5$; (C) effect of different concentrations of **4e** in acetic acid induced writhings in mice; $^a p < 0.001$ compared to control, $^b p < 0.01$ compared to aspirin; $n = 5$.

cy based on inhibition of prostaglandin amplification.[15] Compound **4e** showed concentration dependent significant decrease in the paw edema compared to control after 1 h of carrageenan challenge. At a dose of 100 mg/kg bw, **4e** showed edema volume of 25%, while at 200 mg/kg bw it showed edema of only 15%. Similarly, control and standard diclofenac group showed 33% and 26% edema, respectively. An increase in edema was observed in all groups at 2 and 3 h. A similar trend of activity was observed for **4e** at 1, 2, and 3 h time intervals. At a dose of 200 mg/kg bw, **4e** showed activity comparable to Diclofenac (10 mg/kg bw) at all the observed time intervals, indicating it to be a good candidate for anti-inflammatory activity.

The effect of **4e** was also studied on oxazolone-induced ear edema, which is a simple method for screening systemic and topical anti-inflammatory agents. At a dose of 100 and 200 mg/kg, **4e** showed an edema volume of only 7% and 6%, respectively, compared to control, which showed an edema of 17% (Fig. 5B).

Since most of the anti-inflammatory compounds possess analgesic properties, effect of **4e** on acetic acid-induced writhing in mice was studied using aspirin as positive standard and the results are depicted in Figure 5C. At a dose of 100 and 200 mg/kg bw, **4e** showed 45 and 12 acetic acid-induced writhings in 20 min compared to control and aspirin, which showed 82 and 17 writhings/20 min, respectively. Both the tested doses of **4e** showed significant ($p < 0.001$) decrease in writhings compared to control. At a dose of 200 mg/kg bw, **4e** was superior to aspirin in reducing writhings indicating a good potential for analgesic activity.

3. Conclusions

Synthesis of ninhydrin adducts with phenols as reported was achieved and the best compound, **4e**, possessing free radical scavenging and 5-LOX inhibition was identified. Further studies on **4e** showed it to possess good rate constant toward various free radicals, viz., DPPH, $ABTS^{\bullet-}$, and $^{\bullet}OH$. It was also found to be a potent inhibitor of lipid peroxidation, anti-inflammatory, and analgesic. All these warrant further structural optimization of **4e** for optimum anti-inflammatory and analgesic activity.

4. Experimental

4.1. General

Melting points were determined using Shimadzu Differential Scanning Calorimeter (DSC-60). IR spectra were recorded on a Shimadzu FT-IR 8300 infrared spectrophotometer. Mass spectra were measured on a GCMS-QP5050A spectrometer at 70 eV using direct insertion probe. Elemental analysis was carried out at Central Drug Research Institute, Lucknow, India. The IUPAC names for the structures were obtained using the ACD/I-Lab Web service *(ACD/IUPAC Name 8.05)*. Linoleic acid, arachidonic acid, 2,2-di (4-*tert*-octylphenyl)-1-picrylhydrazyl (DPPH), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), *N*,*N*,*N*′,*N*′,-tetramethyl-*p*-phenylenediamine (TMPD), oxazolone and carrageenan were purchased from Sigma (St. Louis, MO, USA). All the other chemicals were purchased from Qualigens Fine chemicals and were of analytical grade.

4.2. Synthesis

The substrates **2a**–**e** (4.0 mmol) were added to the solution of ninhydrin (250 mg) in 10 ml acetic acid and the mixtures were stirred at 40 °C for 15–30 min and kept at room temperature for 30–60 min, until the solid product separated.[4] The products were filtered, washed with acetic acid and water. The crude products were then recrystallized using petroleum ether–acetone as solvent to yield the following pure analogues.

4.2.1. 4b,6,9b-Trihydroxy-4b,9b-dihydro-10*H*-benzo[*b*]indeno[2,1-*d*]furan-10-one (4a)[4]. Off white solid (0.79 g, 68%); mp 274 °C; IR (KBr) ν_{max}/cm^{-1} 3332, 1701; ^1H NMR (400 MHz in acetone-d_6): δ 8.08 (2H, m), 7.95 (2H, m), 6.81 (1H, t, $J = 6.9$ Hz), 6.59 (1H, d,$J = 8.4$ Hz), 6.56 (1H, d, $J = 8.4$ Hz); Anal. Calcd for $C_{15}H_{10}O_5$: C, 66.67; H, 3.73; Found: C, 64.89; H, 3.52.

4.2.2. 4b,7,9b-Trihydroxy-4b,9b-dihydro-10*H*-benzo[*b*]indeno[2,1-*d*]furan-10-one (4b)[4]. Off white solid (0.67 g, 69%); mp 237 °C; IR (KBr) ν_{max}/cm^{-1} 3224, 1704; ^1H NMR (400 MHz in acetone-d_6): δ 7.96 (1H, br d), 7.84 (1H, br t), 7.73 (1H, br d), 7.64 (1H, br t), 7.25 (1H, d, $J = 8.4$ Hz), 6.43 (1H, dd, $J = 8.4$, 1.9 Hz), 6.25 (1H, d, $J = 1.9$ Hz); Anal. Calcd for $C_{15}H_{10}O_5$: C, 66.67; H, 3.73; Found: C, 65.29; H, 3.68.

4.2.3. 4b,7,9b-trihydroxy-8-methyl-4b,9b-dihydro-10*H*-benzo[*b*]indeno[2,1-*d*]furan-10-one (4c)[4]. White solid (0.87 g, 72%); mp 248 °C; IR (KBr) ν_{max}/cm^{-1} 3376, 3324, 1712; ^1H NMR (400 MHz in acetone-d_6): δ 7.89 (1H, d, $J = 7.8$ Hz), 7.81 (1H, t,$J = 7.2$ Hz), 7.67 (1H, d, $J = 7.4$ Hz), 7.57 (1H, t, $J = 7.6$ Hz), 6.18 (1H, s), 5.98 (1H, d, $J = 1.9$ Hz), 4.14 (2H, br s), 2.35 (3H, s); Anal. Calcd for $C_{16}H_{12}O_5$: C, 67.60; H, 4.25; Found: C, 66.92; H, 4.13.

4.2.4. 4b,6,7,9b-tetrahydroxy-4b,9b-dihydro-10*H*-benzo-[*b*]indeno[2,1-*d*]furan-10-one (4d)[4]. Off white solid (0.85 g, 71%); mp 257 °C; IR (KBr) ν_{max}/cm^{-1} 3554, 3452, 1702; ^1H NMR (400 MHz in MeOH-d_4): δ 7.84 (2H, br s), 7.70 (2H, br s), 6.78 (1H, d, $J = 8.4$ Hz), 6.40 (1H, d, $J = 8.4$ Hz); Anal. Calcd for $C_{15}H_{10}O_6$: C, 62.94; H, 3.52; Found: C, 61.94; H, 3.51.

4.2.5. 4b,11b-dihydroxy-4b,11b-dihydro-12*H*-indeno[1,2-*b*]naphtho[2,3-*d*]furan-12-one(4e)[4]. Light green solid (0.535 g, 42%); mp 214 °C; IR (KBr) ν_{max}/cm^{-1} 3450, 3398, 1695;^1H NMR (400 MHz in acetone-d_6): δ 8.10 (1H, d, $J = 6.6$ Hz), 7.96 (1H, d, $J = 5.4$ Hz), 7.84–7.80 (2H, m), 7.73 (1H, d, $J = 6.6$ Hz), 7.56 (2H, m), 7.45 (3H, m); MS *m*/*z* (%) 275 (6.50), 258 (12.84), 247 (3.71), 231 (10.10), 218 (0.87), 202 (16.64), 189 (4.30), 171 (100.0), 165 (1.81), 143 (14.68), 126 (4.95), 115 (23.88), 107 (0.14), 88 (6.77), 77 (8.75), 63 (2.54), 51

(3.98). Anal. Calcd for $C_{19}H_{12}O_4$: C, 74.99; H, 3.97; Found: C, 75.12; H, 3.91.

5. Free radical reactions

5.1. TLC screening for free radical scavengers

TLC was used for screening the synthesized compounds **4a**–**4e** as radical scavengers. The compounds were dissolved in methanol and spotted onto a Silica gel G F_{254} precoated plates and were developed using 0.5% MeOH and 95% $CHCl_3$ as developing solvent. The plates after development were air-dried and sprayed with DPPH solution (15 mg of DPPH dissolved in 50 ml MeOH) for detecting the compounds with rapid scavenging properties.[16] The compounds that show white spots on a purple background most rapidly were considered as active and were chosen for further investigations.

5.2. 5-Lipoxygenase (5-LOX) and cycloxygenase (COX) inhibition

For lipoxygenase inhibition assay, the 5-LOX was isolated and purified from potato tubers as described by Redanna et al.[17]

The assay was carried out according to the protocol described by Shimizu et al.[18] Briefly, final reaction mixture containing 0.1 mM potassium phosphate buffer, 5-LOX, and the inhibitors (**4a**–**e**) at various concentrations (1–100 μM) was incubated for 5 min. The reaction was started by the addition of 100 μM linoleic acid and the conjugated dienes formed were measured spectrophotometrically at 235 nm. The IC_{50} for the inhibition of 5-LOX was evaluated.

Protocols as described by Madhava Reddy et al.[19] and Copeland et al.[20] were followed for the preparation and purification of COX I and COX II enzyme, and assay for their inhibition. Briefly, the assay mixture contained Tris–HCl buffer (pH 8.0, 100 mM), hematin (15 mM), EDTA (3 mM), enzyme (COX-1 OR COX-2, 100 mg), and test compounds **4a**–**e** (1–100 mM). The mixture was then preincubated at 25 °C for 15 min and then the reaction was initiated by the addition of arachidonic acid (100 mM in 5 ml of ethanol) and TMPD (120 mM in 3 ml of ethanol) in a total volume of 1.0 ml. The enzyme activity was measured by estimating the initial velocity of TMPD oxidation for the first 25 s of the reaction following the increase in absorbance at 603 nm. A low rate of non-enzymatic oxidation observed in the absence of COX-1 and COX-2 was subtracted from the experimental value while calculating the percent inhibition. The effect of different concentrations of indomethacin, celecoxib, and rofecoxib (known inhibitors of COX-1 and COX-2) under the same experimental conditions was also employed for comparative evaluation.

5.3. Reaction with stable DPPH radical

The reaction of DPPH with **4e** was followed by steady-state and time-resolved methods. For steady-state measurements, 100 μM DPPH in methanol was mixed with **4e** (20–220 μM) in methanol and kept in dark for 20 min. The absorbance at 517 nm was monitored both in the presence and absence of **4e**.[21]

5.4. Reaction with $ABTS^{\bullet-}$

The reaction of $ABTS^{\bullet-}$ with **4e** was also followed by steady-state and time-resolved methods. For steady-state measurements, 100 μM $ABTS^{\bullet-}$ [Produced by the reaction of 2 mM ABTS with potassium persulfate (0.17 mM) in phosphate buffer (pH 7.4, 20 mM)] was mixed with **4e** (2–20 μM), mixed well and the absorbance at 734 nm was monitored in the presence and in the absence of **4e**.[21]

Time resolved studies were carried out using stopped-flow spectrometer using single mixing stopped flow reaction analyzer Model SX 18 MV (Applied Photo Physics, UK) with absorption detection. The dead time of the instrument is 1.3 ms. The solutions of **4e** (300–2800 μM) were mixed with methanolic solution of DPPH (100 μM) or equivmolar concentrations of $ABTS^{\bullet-}$ (50–360 μM) and the time-dependent absorbance changes were monitored at 517 and 635 nm, respectively, for DPPH and $ABTS^{\bullet-}$. The analysis of the kinetic traces was carried out with an exponential function using built-in software. The observed rate constant was plotted against molar concentrations of **4e** and the bimolecular rate constant was evaluated from the slope.[22]

5.5. Reaction with $^{\bullet}OH$

Steady-state hydroxyl radical scavenging as measured by degradation of Deoxy-D-ribose method was performed on the aqueous solution of **4e** (2–20 μM) as described by Elizabeth Kunchandy et al.[23] Briefly, Final reaction mixture containing deoxyribose (3 mM), ferric chloride (0.1 mM), EDTA (0.1 mM), hydrogen peroxide (2 mM) in phosphate buffer, pH 7.8 (20 mM) was added with different concentrations of **4e** (2–20 μM) to give a final volume of 3 ml. After incubation for 30 min at ambient temperature, trichloroacetic acid (0.5 ml, 5%) and thiobarbituric acid (0.5 ml, 1%) were added. The reaction mixture was then kept in a boiling water bath for 30 min, cooled, and absorbance was measured at 534 nm. The % inhibition of $^{\bullet}OH$ scavenging with respect to control was calculated and was plotted as a linear plot against different concentrations of **4e** and from the regression equation IC_{50} was calculated.

Hydroxyl radical reactions of different concentrations of **4e** (0.5–5 μM) were also carried out using pulse radiolysis technique employing high-energy electron pulses (50 ns, 7 MeV) obtained from a linear electron accelerator and the transients detected by kinetic spectrometry. Detailed description of the pulse radiolysis setup is given in references.[24] The radiation dosimetry was done using aerated aqueous solution of 0.01 M potassium thiocyanate (KSCN). The dose per pulse was fixed at 19.5 Gy. Radiolysis of water leads to the formation of three highly reactive species namely hydrogen atom ($H^{\bullet}$), hydroxyl radical ($^{\bullet}OH$) and hydrated electron (e^-_{aq}). In N_2O-satu-

rated water, the e^-_{aq} is quantitatively converted into ·OH. $SO_4{}^{\cdot -}$ radicals were generated by the reaction of 0.08 M $S_2O_8{}^{-2}$ with e^-_{aq} at pH 7 and ·OH radicals were scavenged by using 0.1 M *tert*-butanol.

5.6. In vitro lipid peroxidation assay

Egg phosphatidylcholine (20 mg) in chloroform (2 ml) was dried under vacuum in a rotary evaporator to give a thin homogeneous film, and further dispersed in normal saline (5 ml) with a vortex mixer. The mixture was sonicated to get a homogeneous suspension of liposomes.

Lipid peroxidation was initiated by adding 0.05 mM ascorbic acid to a mixture containing liposome (0.1 ml), 150 mM potassium chloride, 0.2 mM ferric chloride, and **4e** (0.75–4.5 mM) in a total volume of 0.4 ml. The reaction mixture was incubated for 40 min at 37 °C. After incubation, the reaction was terminated by adding 1 ml of ice-cold 0.25 M hydrochloric acid containing 20% w/v trichloroacetic acid, 0.4% w/v of thiobarbituric acid, and 0.05% w/v butylated hydroxytoluene. After heating at 80 °C for 20 min, the samples were cooled and the pink chromogen was extracted with a constant amount of butan-1-ol, and the absorbance of the upper organic layer was measured at 532 nm.[25]

6. Pharmacology

6.1. General

All experiments were carried out on inbred Swiss albino mice and Wister rats of both sexes from the animal colony of Kasturba Medical College, Manipal. The colony was maintained under controlled conditions of temperature (23 ± 2 °C) and humidity (50 ± 5%) and a 12-h light–dark cycle. The animals were housed in sanitized polypropylene cages containing sterile paddy husk as bedding. They had free access to standard mouse food and water. All the studies conducted were approved by the Institutional Ethical Committee, Kasturba Medical College, Manipal, according to prescribed guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India. Different doses of **4e**, Aspirin, and Diclofenac were prepared as suspension using 0.5% CMC.

6.2. Acute toxicity studies

Graded doses (1200–2000 mg/kg bw) of **4e** were administered orally to various groups each containing ten mice. On the first day, the animals were evaluated every 10 min for 4 h followed by 24, 48, and 72 h for any changes in spontaneous motor activity, gait, respiration, writhing, piloerection, etc., and up to 30 days for any mortality. The percentage mortality at 24, 72 h, and 30th day was converted into probit values and the LD_{50} was calculated.[26]

6.3. Analgesic and anti-inflammatory studies

6.3.1. Paw edema method. The initial hind paw volume of rats was determined volumetrically by using manual plethysmometer. One percent solution of carrageenan in saline (0.1 ml/rat) was injected subcutaneously into the right hind paw 30 min after **4e** (100 and 200 mg/kg bw) had been administered orally. The animals in the control group received vehicle only. Paw volumes were measured up to 3 h at intervals of 60 min and percent increase in edema between the control and treated groups were compared. Diclofenac (10 mg/kg bw) was used as positive control.[27]

6.3.2. Ear edema method. Different doses of **4e** (100 and 200 mg/kg bw), Diclofenac (10 mg/kg bw), and 0.5% CMC alone (Vehicle control) were administered 1 h prior to the application of oxazolone. Mice were anesthetized using anesthetic ether and the initial thickness of the right ear-lobe was measured using screw gauze and then 0.01 ml of oxazolone (2% solution in acetone) was applied to the inside of the right ear-lobe of both control and treated groups. After 24 h, the mice were again anesthetized and the increase in the thickness of the right ear lobe was measured. The percent increase in edema between the control and treated groups was compared.[28]

6.3.3. Writhing test. The acetic acid-induced abdominal writhing test was performed as described by Whittle.[29] Vehicle, aspirin (100 mg/kg bw), and **4e** (100 and 200 mg/kg bw) were orally administered to mice 30 min before injecting 0.1 ml/10 g of 0.7% acetic acid–saline. The frequency of writhing in mice was counted for the next 20 min.

6.4. Statistics

All data are expressed as means ± SEM. The level of statistical significance was determined by one-way ANOVA between the groups followed by Tukey's test using Graph PAD Instat, Software, USA.

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