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Antioxidant, anti-inflammatory and anti-hyperglycaemic activities of heterocyclic homoprostanoid derivatives

S. A. Manohara Reddy [a,*], Jayesh Mudgal [a], Punit Bansal [a], S. G. Vasanthraju [b], K. K. Srinivasan [c], C. Mallikarjuna Rao [a], N. Gopalan Kutty [a]

[a] Department of Pharmacology, Manipal College of Pharmaceutical Sciences, Manipal University, Madhav Nagar, Manipal 576 104, Karnataka, India
[b] Department of Pharmaceutical Quality Assurance, Manipal College of Pharmaceutical Sciences, Manipal University, Madhav Nagar, Manipal 576 104, Karnataka, India
[c] Department of Pharmaceutical Chemistry, Manipal College of Pharmaceutical Sciences, Manipal University, Madhav Nagar, Manipal 576 104, Karnataka, India

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ABSTRACT

A series of 19 heterocyclic homoprostanoids were synthesized from easily available oleic and ricinoleic acids and evaluated for their possible antioxidant, anti-inflammatory and anti-hyperlipidaemic activities. Compounds with thioxo- and oxoimidazole ring (**1**) and (**2**) have shown potent antioxidant activity with IC_{50} values 0.23 ± 0.09 and 0.41 ± 0.01 mM comparable with standard ascorbic acid. Compound (**3**) with a quinoxaline ring showed maximum inhibition of BSA denaturation at 1 mM concentration and comparable with standard diclofenac. Incorporation of electron withdrawing substitutions like chloro- and nitro-groups in the quinoxaline ring has resulted in an increase anti-inflammatory activity. Test compounds (**3**), (**3a**) and (**3c**) showed modest inhibition of DPP-IV in vitro. However, the unsubstituted quinoxaline (**3**) and substituted quinoxalines (**3b** and **3c**) reduced plasma glucose levels indicating the presence of hypoglycemic activity.

1. Introduction

Inflammation and inflammatory disorders cause a great of morbidity worldwide, resulting in the loss of work hours and cost of treatment. Diseases like atherosclerosis, diabetes and bronchial asthma are now considered to be conditions with a strong inflammatory component.[1] Research is unravelling newer mediators or modulators as responsible for the inflammation. In the treatment of bronchial asthma use is made of the anti-inflammatory effect of inhaled corticosteroids. The statins, which are widely used in dyslipidemia, have anti-inflammatory and antioxidant properties in addition to their lipid-lowering action. Salicylates have been reported to lower the blood glucose level,[2] although they are not considered as treatments for diabetes.

Prostanoids are products of cyclooxygenase biosynthetic pathways and constitute a family of lipidic mediators of widely diverse structures and biological actions. Besides their known pro-inflammatory role, numerous studies have revealed the anti-inflammatory effects of various prostanoids and established their role in the resolution of inflammation. The biological effects of prostanoids depend on the target tissue and the pathophysiological context. Some of these uses are: as healing promoting agents in peptic ulcer, to induce labour along with oxytocin, as abortifacients, in dyslipidemia, in glaucoma,[3] to name a few. The full utilization of the therapeutic potential of prostanoids has not been achieved because of their instability, widespread activity, adverse effects and lack of selectivity.[4]

However, prostanoids may have widely diverse structures. In the present work it was thought worthwhile to prepare some heterocyclic homoprostanoid derivatives with attention at the cyclopentane ring, carboxyl and hydroxyl groups and to subject the synthesized compounds to screening for possible anti-inflammatory, anti-hyperglycaemic and antioxidant effects.

2. Results and discussion

Schemes 1 and 2 summarize the synthetic routes of heterocyclic homoprostanoid derivatives synthesized from oleic or ricinoleic acid. Among the compounds tested for antioxidant activity using DPPH method, Compounds (**1**) and (**1b**) showed good antioxidant activity with IC_{50} values of 0.23 ± 0.09 and 0.23 ± 0.05 mM, respectively. However, the known antioxidant ascorbic acid exhibited an IC_{50} of 0.40 ± 0.07 mM (Table 1). Inhibition of the denaturation of bovine serum albumin (BSA) is a quick screening method for anti-inflammatory activity. In this test, Compounds (**1**– **3**) and

* Corresponding author. Tel.: +91 99860 95583.
E-mail addresses: manohar_sou@yahoo.co.in, manoharsou@gmail.com (S.A. Manohara Reddy).

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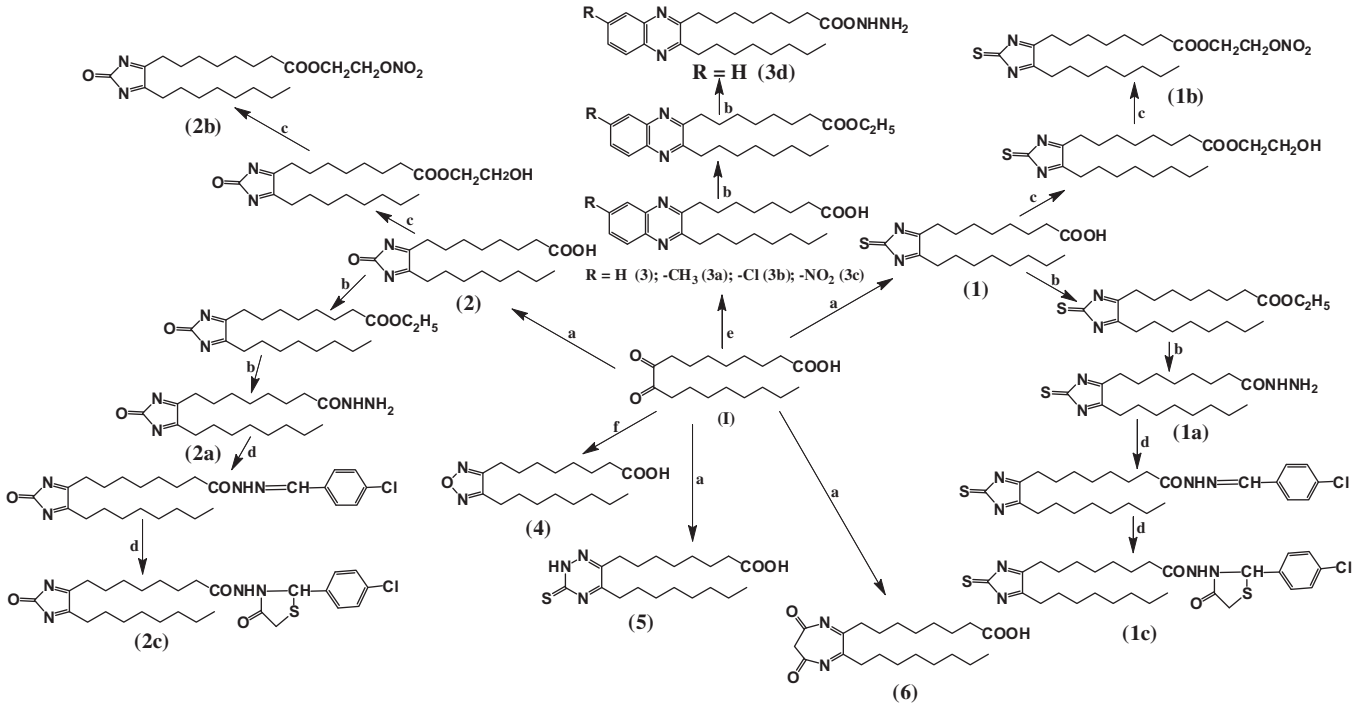
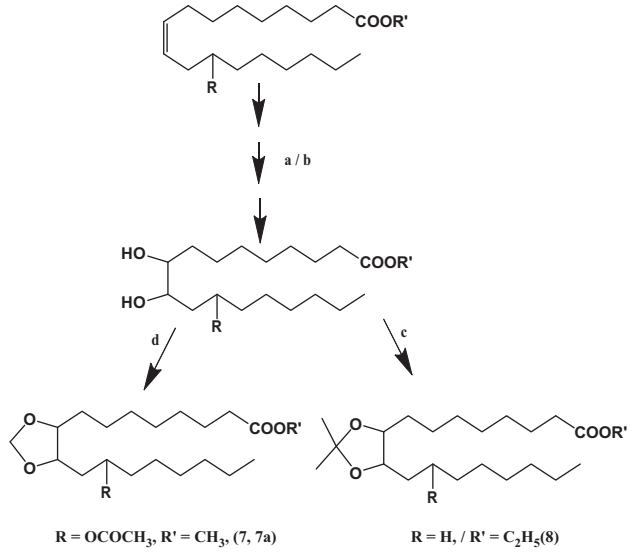


Scheme 1. Reagents and conditions: (a) thiourea/urea/thiosemicarbazide/malonamide, pyridine, dilute HCl, 8/12 h; (b) ethanol, concn H_2SO_4, 3 h, hydrazine hydrate (99%), 7 h; (c) ethylene diol, dry benzene, *p*-TsOH, 12 h, HNO_3 (70%), H_2SO_4 (95%), 0 °C, 1 h; (d) *p*-chlorobenzaaldehyde, methanol, glacial acid, 45–60 min, mercaptoacetic acid (98%), 24 h; (e) *o*-phenylenendiamine/4-chloro-*o*-phenylenediamine/4-nitro-*o*-phenylenediamine, ethanol, glacial acid, 1 h; (f) hydroxylamine HCl, ethanol, pyridine, 1 h.



Scheme 2. Reagents and conditions: (a) pyridine, acetyl chloride, 1 h, methanol, *p*-TsOH, 4 h, gl.a.acid, concn H_2SO_4, H_2O_2 (28%), 4 h, 40 °C; (b) KOH, $KMnO_4$, sodium thiosulfate, concn HCl (37%), ethanol, 3 h; (c) dimethoxy propane, acetone, *p*-TsOh, 3 h; (d) *p*-formaldehyde, orthophosphoric acid (85%), 4 h.

(**3b**) showed significant inhibition of BSA denaturation. The standard drug, diclofenac, showed 56.27 ± 1.90 (1 mM), 49.46 ± 1.09 (0.5 mM), 42.55 ± 0.90 (0.25 mM) and 38.29 ± 5.90 (0.01 mM) percent inhibition, respectively. Values more than 25% inhibition are significant (p <0.05). The activity of these compounds might be due to electrostatic forces and hydrophobic bonding involved in the stabilization of albumin against denaturation[5] (Table 1). The presence of chloro (**3b**)/methyl (**3a**)/nitro (**3c**) group on quinoxaline ring may have resulted in lowering the activity.

Inhibition of DPP-IV is a measure of the possible in vitro antidiabetic effect of a molecule. Compounds (**3**) and (**3a**) were the most active in this test and showed 73.56% and 88.08% DPP-IV inhibition, respectively (Table 2).

Test compounds (**3**), (**3b**) and (**3c**) (100 mg/kg/po) produced significant (p <0.05), that is, 34.38 ± 10.09, 26.74 ± 3.78 and 39.58 ± 4.83% inhibition of paw edema caused by carrageenan injection in rats (Table 3). Compounds (**3a**) and (**6**) were found to be less active. The lack of activity in the present study might be due to reduced or lack of oral absorption. However, Compounds (**1b**) and (**2b**) were synthesized with a view to producing nitric oxide releasing compounds. Nitric oxide exerts pro as well as anti-inflammatory activity at different conditions. It was thought that incorporating a nitric oxide releasing ester function would impart anti-inflammatory activity to the compound. But, in the present study this was not found to do so. However, quinoxaline derivatives (**3**, **3b** and **3c**) exhibited significant activity. The unsubstituted quinoxaline compound, which showed modest anti-inflammatory activity in the earlier work,[6] has also proved to be effective in the present study. Incorporation of electron withdrawing substitutions like chloro- or nitro-groups in the quinoxaline ring has resulted in an increased anti-inflammatory activity. Substitution of the methyl group at the same position (+ I effect) has resulted in the loss of activity (Table 3).

In Freund's complete adjuvant (FCA) induced chronic inflammation in rats, Compounds (**3b**) and (**3c**) (100 mg/kg/po) produced a maximum % inhibition, that is, 44.22 ± 06.23 and 53.33 ± 18.70 on day 35, which was significant (p <0.05) compared with FCA control (0.90 ± 0.08). Diclofenac (5 mg/kg/po) also showed significant (p <0.05) percent inhibition of the foot volume, that is, 35.27 ± 8.86, compared to FCA control, see Figure 1.

Among the compounds tested for inhibition of myeloperoxidase induced by FCA, compounds (**3c**) showed 182.81 ± 12.89 percentage activity of MPO, which was statistically significant (p <0.05) when compared with FCA control (i.e., 282.16 ± 17.60). Standard

Table 1
In vitro antioxidant and anti-inflammatory activity of heterocyclic homoprostanoids against DPPH radical scavenging and bovine serum albumin denaturation, data are expressed as means ± SEM (n = 6)

Test compounds	DPPH method IC_{50} values (mM)	Inhibition of BSA denaturation (%)	Concentrations (mM)
1	0.23 ± 0.09	78.30 ± 0.00	1
1a	0.44 ± 0.09	37.23 ± 0.12	0.01
1b	0.23 ± 0.05	38.07 ± 0.13	0.01
1c	NT[a]	NT[a]	NT[a]
2	0.41 ± 0.01	76.66 ± 0.97	1
2a	3.87 ± 0.07	31.38 ± 0.00	0.01
2b	3.20 ± 0.05	25.10 ± 0.28	0.01
2c	NT[a]	NT[a]	NT[a]
3	1.33 ± 0.05	75.66 ± 0.02	1
3a	0.61 ± 0.04	37.41 ± 0.00	1
3b	0.99 ± 0.04	57.14 ± 0.17	1
3c	1.11 ± 0.07	36.88 ± 0.00	1
3d	>2	03.72 ± 0.00	0.01
4	>2.5	25.39 ± 0.26	5
5	4.48 ± 0.06	14.00 ± 0.50	0.25
6	>10	37.00 ± 0.66	0.25
7	4.09 ± 0.06	48.53 ± 2.09	0.01
7a	1.80 ± 0.09	54.39 ± 0.28	0.01
8	2.35 ± 0.03	46.44 ± 0.00	5
Ascorbic acid	0.40 ± 0.07	—	—
Diclofenac	—	56.27 ± 1.90	1

[a] NT = not tested.

Table 2
Effect of heterocyclic homoprostanoids derivatives on in vitro DPP-IV enzyme inhibition assay, data are expressed as means ± SEM (n = 6)

Test compounds (concn 500 μM)	% DPP-IV inhibition
1	−43.61 ± 0.92
1a	−37.44 ± 0.57
1b	23.27 ± 8.52
1c	−25.11 ± 1.7
2	−23.34 ± 0.52
2a	−36.56 ± 0.16
2b	−6.22 ± 2.62
2c	−91.18 ± 0.92
3	73.56 ± 5.28
3a	88.08 ± 8.51
3b	45.53 ± 1.70
3c	63.95±0.29
3d	−202.20 ± 0.02
4	02.20 ± 0.88
5	58.29 ± 18.51
6	12.34 ± 0.85

diclofenac treatment also showed 188.93 ± 24.18 percent inhibition of myeloperoxidase, see Figure 2.

None of the compounds showed any effect on OGTT when administered orally, see Figure 3. However, on ip administration, compounds (**3b**) and (**3c**) showed 36.71 ± 10.66 and 45.11 ± 3.00 percentage reductions in glucose levels, see Figure 3. Reduction in glucose level might be due to the presence of nitro group in quinoxaline ring.

3. Conclusion

Compounds (**1**), (**1b**) and (**2**) have shown potent antioxidant activity in DPPH method when compared with standard ascorbic acid in vitro. In in vitro anti-inflammatory activity, Compounds (**1a**), (**1b**), (**2a**), (**2b**), (**7**) and (**7a**) showed modest inhibition of thermal denaturation of albumin and was significant when compared

Table 3
Effect of heterocyclic homoprostanoids derivatives against carrageenan induced acute hind paw edema in rats, data are expressed as means ± SEM (n = 6)

Test compounds	Paw edema (Δ mL)	Inhibition (%)
Carrageenan control	0.72 ± 0.11	0
Diclofenac	0.45 ± 0.06	38.19 ± 8.15
1	0.85 ± 0.11	−7.17 ± 14.00
1b	0.97 ± 0.10	−22.36 ± 12.67
1c	0.96 ± 0.06	−21.73 ± 7.3
2	0.89 ± 0.10	−12.87 ± 12.63
2b	0.97 ± 0.07	−22.78 ± 8.43
2c	0.79 ± 0.04	0.00 ± 4.72
3	0.47 ± 0.07*	34.38 ± 10.09
3a	0.63 ± 0.08	12.50 ± 5.70
3b	0.53 ± 0.03*	26.74 ± 3.78
3c	0.44 ± 0.03*	39.58 ± 4.83
5	0.75 ± 0.16	−3.82 ± 22.76
6	0.71 ± 0.09	1.74 ± 12.65

* $p < 0.05$ as compared to carrageenan control group.

with standard diclofenac. Compounds (**3**), (**3b**) and (**3c**) exhibited statistically significant anti-inflammatory activity in carrageenan-induced paw edema model and were comparable with standard diclofenac and also showed good activity when screened against FCA induced chronic inflammation. The present work has revealed that heterocyclic homoprostanoid derivatives with quinoxaline possess modest antioxidant and anti-inflammatory activities. A thorough structure–activity relationship (SAR) has not been carried out. With more detailed SAR studies, it will be possible to develop molecules that have greater and more selective activity. It will also be possible to develop molecules that have multiple beneficial effects such as anti-hyperlipidaemic and anti-hyperglycaemic activities in the same molecule. A future work could possibly address the lacunae in the current study.

4. Experimental

4.1. Chemistry

All the chemicals and solvents used were purchased from standard chemical suppliers. Ricinoleic acid (88%) pure was obtained from Rayalaseema Alkalies Ltd, Kurnool, India, as a gift sample. Diclofenac sodium was purchased from Sigma–Aldrich, St. Louis, MO, USA. No attempts were made to optimize yields. Melting points were determined on a Toshniwal capillary melting point apparatus and are uncorrected. Thin layer chromatography was carried out on silica gel plates (Merck $60F_{254}$) and the spots were visualized under UV lamp (254 or 365 nm) and/or iodine vapour. IR spectra were recorded on FTIR-8300 Shimadzu and Brucker Tensor 27 using KBr pellet or neat. ^1H NMR and ^{13}C NMR were recorded at 400 and 100 MHz (Brucker), $CDCl_3$ and DMSO-d_6 as solvent. The chemical shifts are reported in parts per million (δ) and signals are quoted as s (singlet), d (doublet), t (triplet), m (multiplet). Mass spectrum was recorded on GC–MS/LC–MS—2010 Shimadzu, Japan (methanol/water; 80:20; acetonitrile/0.1% formic acid; 70:30).

4.1.1. Preparation of 9,10-diketostearic acid (I)[7]

Oleic acid (0.02 mol) was dissolved by stirring in water (3 L) containing KOH (1.3 g). The solution was brought to pH 7.5 by bubbling in CO_2. A solution of potassium permanganate (6.3 g, 0.04 mol) in water (300 mL) was added and the reaction carried out at room temperature. Bubbling in of CO_2 was continued to keep the pH at 7–7.5. After 1 h, the excess of potassium permanganate and the precipitated MnO_2 were destroyed with sodium metabisulphite and HCl. Diketostearic acid, which separated was filtered

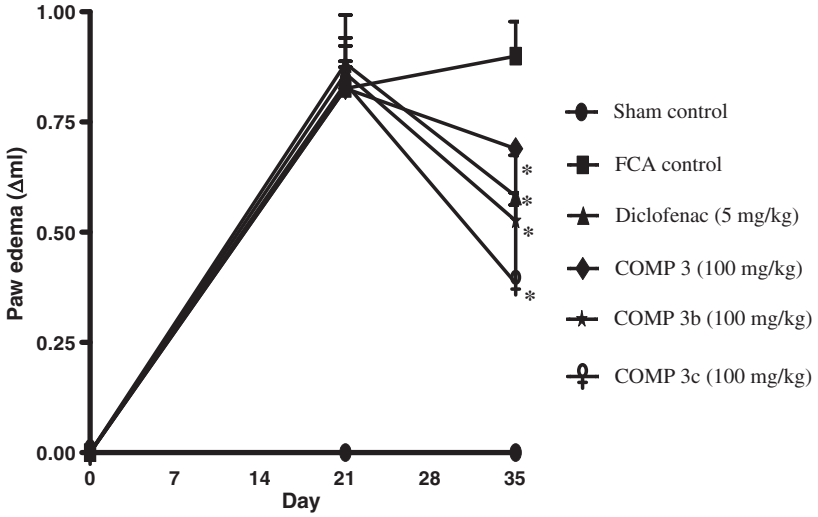


Figure 1. Effect of heterocyclic homoprostanoids derivatives against FCA induced chronic hind paw edema in rats ($n = 6$). $^{*}p < 0.05$ as compared to FCA control group.

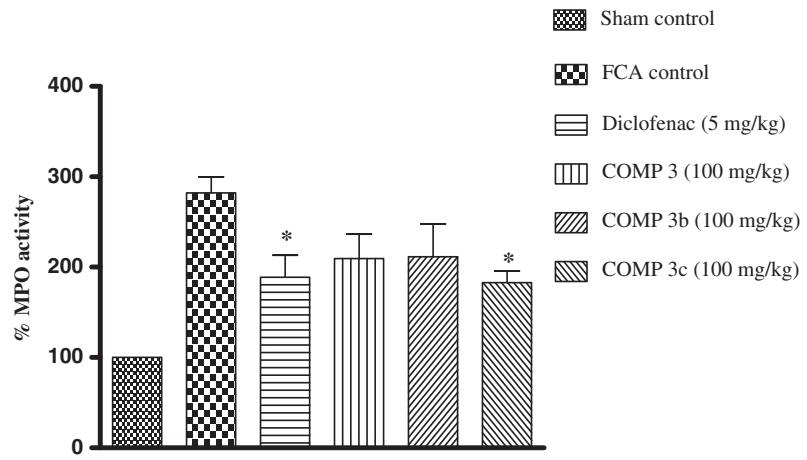


Figure 2. Effect of heterocyclic homoprostanoids derivatives on MPO activity, against FCA induced chronic hind paw edema in rats ($n = 6$). $^{*}p < 0.05$ as compared to FCA control group.

and dried. The crude product was recrystallized from ethanol to give pale yellow needles. Yield: 65%. R_f 0.38 (Hexane/ethyl acetate/acetone; 62:38:01). Mp 82–83 °C. IR (KBr): 3384 (b, –OH of –COOH), 2918 (–CH_2–, asymmetric), 2860 (–CH_2–, symmetric), 1697 (s, –*C*=*O* of α-diketone and –C=O of –*COOH*), 1464, 1382, 1236, 1111, 1074, 937 and 723 cm^{-1}. MS-EI: for $C_{18}H_{32}O_4$, calcd 312.23, found 311.10. *m*/*z* $(M-1)^+$.

4.1.1.1. General procedure for the preparation of compounds (1, 2, 5, 6)[6]. 9,10-Diketo stearic acid (0.01 mol) was refluxed with thiourea (0.01 mol) (**1**)/urea (0.03 mol) (**2**)/thiosemicarbazide (0.01 mol) (**5**)/malonamide (0.01 mol) (**6**) in pyridine for 8–12 h. The cooled mixture was poured onto cold dilute HCl and extracted with ether. The crude product was recrystallized from ethyl acetate to petroleum ether.

4.1.2. General procedure for the preparation of compounds (1a, 2a, 3d)

4.1.2.1. Step-1: preparation of 8-(5-octyl-2-thioxo-2*H*-imidazol-4-yl)octanoic acid ethyl ester/ethyl 8-(5-octyl-2-oxo-2*H*-imidazol-4-yl)octanoic acid/ethyl 8-(3-octylquinoxalin-2-yl)octanoic acid. 0.014 mol of compound (**1**)/0.015 mol of compound (**2**) in ethanol (30 mL) was refluxed with H_2SO_4 (2 mL) for 3 h. The mixture was cooled and poured onto crushed ice. Sodium bicarbonate solution was added to remove excess of acid and extracted with ether. After drying over Na_2SO_4, the ether was evaporated to get the ester as a thick liquid.

4.1.2.2. Step-2: preparation of compounds (1a/2a/3d). 0.017 mol of ethyl 8-(5-octyl-2-thioxo-2*H*-imidazol-4-yl)octanoate/0.011 mol of ethyl 8-(5-octyl-2-oxo-2*H*-imidazole-4-yl)octanoate/0.007 mol ethyl 8-(3-octylquin oxalin-2-yl)octanoate was refluxed with 3 mL of hydrazine hydrate (99%) in ethanol (30 mL) for 3 h. The mixture was cooled; separated solid was filtered and dried. The crude product was recrystallized from ethanol.

4.1.3. General procedure for the preparation of compounds (1b/2b)

4.1.3.1. Step-1: preparation of 2-hydroxyethyl 8-(5-octyl-2-thioxo-2*H*-imidazol-4-yl) octanoate/2-hydroxyethyl 8-(5-octyl-2-oxo-2*H*-imidazol-4-yl)octanoate. 0.018 mol of compound (**1**)/0.014 mol of compound (**2**), ethylene glycol (10 mL), 40 mg *p*-TsOH and dry benzene (100 mL) were refluxed for 11 h using a Dean–Stark water separator. Dry benzene (30 mL) was added periodically to replenish the flask. After 12 h the benzene was removed in vacuo and the mixture was taken up in water and extracted with

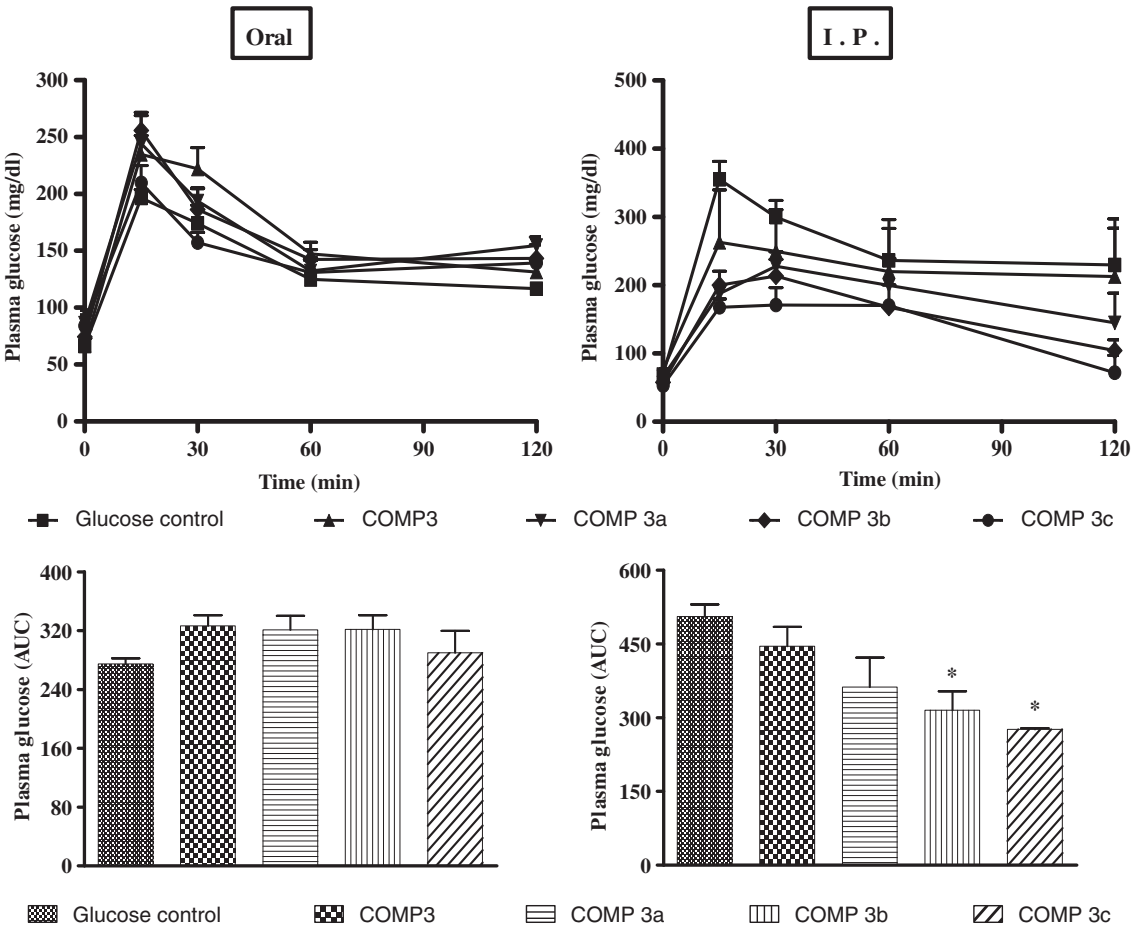


Figure 3. Effect of route of administration of heterocyclic homoprostanoids derivatives on plasma glucose (A) mg/dl (B) AUC, against OGTT in mice ($n = 6$). $^{*}p < 0.05$ as compared to glucose control group.

ether. The ether layer was washed with sodium bicarbonate solution (10%) and then with water and dried over anhydrous Na_2SO_4. Removal of the solvent afforded 2-hydroxyethyl 8-(5-octyl-2-thioxo-2*H*-imidazol-4-yl)octanoate/2-hydroxyethyl 8-(5-octyl-2-oxo-2*H*-imidazol-4-yl)octanoate.

4.1.3.2. Step-2: preparation of compounds (1b/2b). 2-Hydroxy ethyl 8-(5-octyl-2-thioxo-2*H*-imidazol-4-yl)octanoate (0.012 mol) /2-hydroxy ethyl 8-(5-octyl-2-oxo-2*H*-imidazol-4-yl)octanoate (0.013 mol) was added drop wise to a stirred solution of 70% HNO_3 (2.2 mL) and 95% H_2SO_4 (5 mL) at 0 °C with continuous stirring. After complete addition of the ester, stirring was continued for an additional hour. To the resulting suspension, ice cold water was added and it was extracted with ethyl acetate. Ethyl acetate layer was washed with water until aqueous layer was acid free. Removal of ethyl acetate gave a viscous liquid. The crude product was purified on silica gel column (20 g, SiO_2; ethyl acetate/petroleum ether: 10/90).

4.1.4. General procedure for the preparation of compounds (1c/2c)

4.1.4.1. Step-1: preparation of *N′*-benzylidene-8-(5-octyl-2-thioxo-2*H*-imidzol-4-yl) octanehydrazide/*N′*-benzylidene-8-(5-octyl-2-oxo-2*H*-imidzol-4-yl)octanehydrazide. 0.01 mol[8] of compound (**1a**)/0.01 mol of compound (**2a**) and *p*-chloro benzaldehyde (0.01 mol) were dissolved separately in 10 mL of methanol and mixed in a 100 mL R.B. flask. To the mixture a few drops of glacial acetic acid were added and refluxed for 45–60 min. Cooled, filtered the product and washed with a little methanol and dried. The crude product was recrystallized from methanol.

4.1.4.2. Step-2: preparation of compounds (1c/2c). 0.002 mol of *N′*-benzylidene-8-(5-octyl-2-thioxo-2*H*-imidazol-4-yl)octane hydrazide/0.002 mol of *N′*-benzylidene-8-(5-octyl-2-oxo-2*H*-imidazol-4-yl)octane hydrazide was converted to the respective thiazolidin-4-one by following the method of Surrey, using mercaptoacetic acid (0.7 mL) in dry benzene (30 mL) and refluxing along with azeotropic water removal. The crude product was recrystallized from methanol.

4.1.5. General procedure for the preparation of compounds (3, 3a, 3b, 3c)[6]

0.01 mol of 9,10-diketo stearic acid in ethanol was boiled under reflux for 1 h with *o*-phenylene diamine (0.01 mol) (**3**)/4-methyl-*o*-phenylenediamine (0.01 mol) (**3a**)/4-chloro-*o*-phenylenediamine (0.01 mol) (**3b**)/4-nitro-*o*-phenylenediamine (0.01 mol) (**3c**) and a few drops of glacial acetic acid. The reaction mixture was cooled and ethanol was removed under reduced pressure, the resulting mixture was poured into water and extracted with ether. The ether extract was shaken with two successive 20 mL portions of saturated sodium bicarbonate solution. Acidification of the aqueous extract gave a semisolid, extracted with ether and dried over anhydrous sodium sulphate.

4.1.6. Preparation of compound (4)

0.01 mol of 9,10-Diketo stearic acid in ethanol was warmed for 30 min with 0.02 mol of hydroxylamine hydrochloride and a few drops of pyridine. Removal of ethanol yielded the dioxime as a colourless solid. The crude product was recrystallized from aqueous ethanol to give colourless (needles). The dioxime (0.0075 mol) was refluxed in pyridine for 90 min. Cooled and treated with excess of dilute HCl to remove pyridine. The separated solid was filtered and dried. The crude product was recrystallized from aqueous ethanol.

4.1.7. Preparation of methyl 8-(5-(2-acetoxyoctyl)-2,2-dimethyl-1,3-dioxolan-4-yl) octanoate (7)

4.1.7.1. Step-1: preparation of 12-acetylricinoleic acid. 0.05 mol of ricinoleic acid was dissolved in pyridine (15 mL) in a dry 250 mL conical flask and cooled to 0–5 °C in an ice bath. To this acetyl chloride (7 mL) was added dropwise with continuous stirring. After complete addition of acetyl chloride, the reaction mixture was heated on a water bath for 15–20 min to complete the reaction. The mixture was cooled and poured onto ice cold water (200 mL) and acidified with dilute HCl. Then extract with ether, ethereal layer was washed with water and organic solvent was dried over anhydrous magnesium sulphate. Removal of the solvent gave pale yellow viscous liquid. The crude product was purified on silica gel column (SiO_2, 15 g, petroleum ether) to give a pale yellow liquid (Yield: 55%).

4.1.7.2. Step-2: preparation of methyl-12–acetylricinoleic acid. 0.029 mol of 12-acetylricinoleic acid was dissolved in methanol (50 mL), to this 40 mg of *p*-TsOH was added and refluxed for 4 h at 100 °C. The mixture was cooled and poured onto ice cold water (200 mL). A saturated solution of sodium bicarbonate was added to neutralize any unreacted acid. Then mixture was extracted with ether, washed ethereal layer with water and dried over anhydrous magnesium sulphate. Removal of the solvent gave a pale yellow liquid (Yield: 73%). IR (neat); 3276, 3005 (–CH=CH–), 2922 (–CH_2–, asymmetric), 2855 (–CH_2–, symmetric), 1730 and 1700 (*s*, –*C*=*O* of –*COOCH*$_3$ and –*OCOCH*$_3$), 1454, 1373, 1239, 1188, 1140, 926, 845 and 720 cm^{-1}.

4.1.7.3. Step-3: preparation of 9,10-dihydroxymethyl-12-acetylricinoleic acid[9]. To a stirred solution of methyl-12-acetylricinoleic acid (0.019 mol), glacial acetic acid (13.2 mL), containing concn sulphuric acid (0.5 mL), H_2O_2 (28%, 3.5 mL) was added dropwise while maintaining the reaction temperature at 40 °C. The reaction mixture was stirred for additional 4 h at 40 ± 2 °C and left over night at room temperature. Solid $NaHSO_3$ was added with stirring to destroy the excess of H_2O_2. Acetic acid was removed under reduced pressure. The residual solution was poured onto ice cold water, extracted with ether, ethereal layer was washed with water and organic solvent was dried over anhydrous magnesium sulphate. Removal of the solvent gave 9,10-dihydroxymethyl-12-acetylricinoleic acid as a pale yellow liquid (Yield: 65%). IR (neat); 3512 and 3426 (b, –OH), 2925 (–CH_2–, asymmetric), 2857 (–CH_2–, symmetric), 1736 (*s*, –*C*=*O* of –*COOCH*$_3$ and –*OCOCH*$_3$), 1450, 1363, 1246, 1175, 1081 and 1023 (–OH), 859 and 723 cm^{-1}.

4.1.7.4. Step-4: preparation of methyl 8-(5-(2-acetoxyoctyl)-2, 2-dimethyl-1,3-dioxolan-4-yl)octanoate[10]. 9,10-Dihydroxymethyl-12-acetylricinoleic acid (0.011 mol) and paraform aldehyde (3 g) were dissolved together in orthophosphoric acid (50 mL) and the solution was heated at 100 °C for 4 h. The reaction mixture was cooled and extracted with ether. The combined ether extract was concentrated under reduced pressure. The crude product was purified on silica gel column (SiO_2, 15 g, EtoAc/petroleum ether; 30/70) to give methyl 8-(5-(2-acetoxyoctyl)-2, 2-dimethyl-1,3-dioxolan-4-yl)octanoate as a pale brownish liquid.

4.1.8. Preparation of acetonide of 9,10–dihydroxy-methyl-12-acetylricinoleic acid (7a)[10]

To a solution of 9,10-dihydroxy-methyl-12-acetylricinoleic acid (0.01 mol) in a mixture of dimethoxypropane and acetone (1/1, 70 mL) was added *p*-TsOH (0.016 mol). After 3 h stirring at room temperature, the reaction was neutralized with sodium hydrogen carbonate solution and concentrated in vacuo. The crude product was purified on a silica gel column (SiO_2, 30 g, EtoAc/petroleum ether; 5/95) to obtain the acetonide of 9,10-dihyrdoxy-methyl-12-acetylricinoleic acid.

4.1.9. Preparation of acetonide of 9,10-dihydroxy stearic acid ethyl ester (8)

Compound (**8**) was prepared as reported in the earlier paper.[10]

4.1.10. 8-(5-Octyl-2-thioxo-2*H*-imidazol-4-yl)octanoic acid (1)

Colourless solid. Yield: 35%. R_f 0.50 (Hexane/ethyl acetate/acetone; 62:38:01). Mp 121–122 °C. IR (KBr): 3400–2500 (b, –OH of –COOH), 2924 (–CH_2–, asymmetric), 2852 (–CH_2–, symmetric), 1697 (s, –C=O of –COOH), 1610 (–C=N), 1288 (–C=S), 1070 (–C–N), 1003, 949, 819, 754 and 665 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$), δ: 0.89 (t, 3H, CH_3), 1.20–1.40 (m, 16H, $(CH_2)_n$), 1.53–1.68 (m, 6H, $(CH_2)_n$), 2.30–2.48 (m, 2H, CH_2COOH), 8.85 (s, 1H, COOH) ppm. ^{13}C NMR (100 MHz, DMSO-d_6), δ: 14.90 (CH_3), 24.15, 24.87, 26.49, 27.45, 30.17, 30.36, 30.58, 30.77, 31.16, 33.44, 33.98, 34.32, 34.53, 35.42, 128.39, 131.40 (C-9, C-10), 158.27 (*C*=*S*), 178.14 (COOH) ppm. MS-CI: for $C_{19}H_{32}N_2O_2S$, calcd 352.22, found 352 *m*/*z* $(M)^+$; MS-EI: 353.20 *m*/*z* $(M+1)^+$.

4.1.11. 8-(5-Octyl-2-thioxo-2*H*-imidazol-4-yl)octanehydrazide (1a)

Colourless solid (needles). Yield: 75%. R_f 0.40 (ethyl acetate/methanol; 20:80). Mp 110–114 °C. IR (KBr): 3317 (s, –NH_2), 3178, 2918 (–CH_2–, asymmetric), 2848 (–CH_2–, symmetric), 1630 (s, –C=O of –*CONHNH*$_2$), 1465 (–C=N), 1323 (–C=S), 1161, 1014 (–C–N), 950, 770, 725 and 617 cm^{-1}. MS-EI: for $C_{19}H_{34}N_4OS$, calcd 366.25, found 365 *m*/*z* $(M-1)^+$.

4.1.12. 2-(Nitrooxy)ethyl 8-(5-octyl-2-thioxo-2*H*-imidazol-4-yl)octanoate (1b)

Pale brownish liquid. Yield: 57%. IR (neat); 3147, 2923 (–CH_2–, asymmetric), 2857 (–CH_2–, symmetric), 1707 (s, –C=O of –*COOCH*$_2$*CH*$_2$*ONO*$_2$), 1639 (–C=N), 1555 and 1457 (–ONO_2, asymmetric and symmetric), 1275 (–C=S), 1104, 1053 (–C–N), 1008, 904, 851, 723 and 638 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$), δ: 0.91 (t, 3H, CH_3), 1.20–1.40 (m, 16H, $(CH_2)_n$), 1.60–1.70 (m, 6H, $(CH_2)_n$), 2.35 (m, 2H, CH_2COO–), 4.2–4.75 (m, 4H, –*COOCH*$_2$*CH*$_2$–) ppm. MS-EI: for $C_{21}H_{35}N_3O_5S$, calcd 441.23, found 440 *m*/*z* $(M-1)^+$.

4.1.13. *N*-(2-4-Chlorophenyl)-4-oxothiazolidin-3-yl)-8-(5-octyl-2-thioxo-2*H*-imidazol-4-yl)-octanamide (1c)

Colourless solid. Yield: 13%. R_f 0.79 (ethyl acetate/methanol; 20:80). Mp 142–146 °C. IR (KBr); 3443 (–NH), 3036 (–CH–, aromatic), 2914 (–CH_2–, asymmetric), 2850 (–CH_2–, symmetric), 1685 (s, –C=O of –CONH–), 1589 (aromatic), 1485 (–C=N), 1398, 1294 (–C=S), 1220, 1165, 1085 (–C–N), 1008, 933, 831, 763, 646 (–*C*–*S*–*CH*$_2$) and 516 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$), δ: 3.70–3.90 (d, 2H, *S*–*CH*$_2$–*CO*), 5.9 (s, 1H, CH), 7.30–7.55 (m, 4H, aromatic), 8.57 (br s, 1H, NH) ppm. MS-EI: for $C_{28}H_{39}ClN_4O_2S_2$, calcd 562.22, found 562 *m*/*z* $(M)^+$.

4.1.14. 8-(5-Octyl-2-oxo-2*H*-imidazol-4-yl)octanoic acid (2)

Pale yellow solid. Yield: 37%. R_f 0.45 (ethyl acetate/methanol; 12:88). Mp 118–122 °C. IR (KBr); 3400–2500 (b, –OH of –COOH), 2918 (–CH_2–, asymmetric), 2858 (–CH_2–, symmetric), 1699 (s, –C=O of –COOH), 1597 (–C=N), 1095 (–C–N), 1062, 970, 842,

736 and 675 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$), δ: 0.90 (t, 3H, CH_3), 1.20–1.37 (m, 18H, $(CH_2)_n$), 1.45–1.65 (m, 6H, $(CH_2)_n$), 2.35 (m, 2H, CH_2COOH), 7.60 (s, 1H, COOH) ppm. ^{13}C NMR (100 MHz, DMSO-d_6), δ: 13.83 (CH_3), 22.01, 23.85, 24.40, 25.87, 28.43, 31.12, 33.61, 38.96, 39.16, 39.37, 39.58, 39.78, 39.99, 122.89, 136.22 (C-9, C-10), 148.93 (*C*=*O*), 174.47 (COOH) ppm. MS-CI: for $C_{19}H_{32}N_2O_3$, calcd 336.47, found 337 m/z (M+1) $^+$; MS-EI: 337.30 m/z $(M+1)^+$.

4.1.15. 8-(5-Octyl-2-oxo-2*H*-imidazol-4-yl)octanehydrazide (2a)

Colourless solid (needles). Yield: 40%. R_f 0.78 (chloroform/methanol; 80:20). Mp 112–116 °C. IR (KBr); 3317 (–NH), 3178, 3041 (–CH–, aromatic), 2918 ($-CH_2-$, asymmetric), 2848 ($-CH_2-$, symmetric), 1629 (s, –C=O of –CONH–), 1533, 1464 (–C=N), 1089 (–C–N), 949, 771, 725 and 617 cm^{-1}. MS-EI: for $C_{19}H_{34}N_4O_2$, calcd 350.27, found 348 m/z $(M-2)^+$.

4.1.16. 2-(Nitrooxy)ethyl 8-(5-octyl-2-oxo-2*H*-imidazol-4-yl)octanoate (2b)

Pale brownish liquid. Yield: 47%. IR (neat); 3200, 2926 ($-CH_2-$, asymmetric), 2859 ($-CH_2-$, symmetric), 1712 (s, –C=O of –*COOCH*$_2$*CH*$_2$–), 1640 (–C=N), 1562 and 1455 ($-ONO_2$, asymmetric and symmetric), 1376, 1274, 1172, 1108 (–C–N), 900, 852, 759, 724 and 629 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$), δ: 0.89 (t, 3H, CH_3), 1.20–1.40 (m, 16H, $(CH_2)_n$), 1.60–1.99 (m, 6H, $(CH_2)_n$), 2.35 (m, 2H, CH_2COO-), 3.7–4.79 (m, 4H, $COOCH_2CH_2-$) ppm. MS-EI: for $C_{19}H_{34}N_4O_2$, calcd 425.25, found 425 m/z $(M)^+$.

4.1.17. *N*-(2-4-Chlorophenyl)-4-oxothiazolidin-3-yl-8-(5-octyl-2-oxo-2*H*-imidazol-4-yl)-octanamide (2c)

Colourless solid. Yield: 10%. R_f 0.65 (ethyl acetate/methanol; 20:80). Mp 139–142 °C. IR (KBr); 3394 (–NH), 3090 (–CH–, aromatic), 2918 ($-CH_2-$, asymmetric), 2850 ($-CH_2-$, symmetric), 1690 and 1651 (s, –C=O of –CONH– and –CO–CH_2–S–), 1590, 1465 (–C=N–), 1309, 1274, 1087 (–C–N–), 970, 842, 736, 675 and 582 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$), δ: 3.73–3.90 (*d*, 2*H*, *S*–*CH*$_2$–*CO*), 6.1 (s, 1H, CH), 7.25–7.55 (m, 4H, aromatic), 8.58 (br s, 1H, NH) ppm. MS-EI: for $C_{28}H_{39}ClN_4O_3S$, calcd 546.24, found 547 m/z $(M+1)^+$.

4.1.18. 8-(3-Octylquinoxalin-2-yl)octanoic acid (3)

Pale yellowish brown solid. Yield: 45%. R_f 0.43 (ethyl acetate/methanol; 12:88). Mp 58–60 °C. IR (KBr): 3400–2500 (b, –OH of –COOH), 3061 (–CH–, aromatic), 2926 ($-CH_2-$, asymmetric), 2852 ($-CH_2-$, symmetric), 1730 (s, –C=O of –COOH), 1566 (–*C*=*N*), 1103 (–C–N), 962, 858, 765 and 657 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$), δ: 0.90 (t, 3H, CH_3), 1.26–1.48 (m, 16H, $(CH_2)_n$), 1.60–1.70 (m, 4H, $(CH_2)_n$), 1.86 (m, 2H, CH_2), 2.35 (m, 2H, CH_2COOH), 3.0 (m, 4H, $(CH_2)_2$), 7.63–7.80 (m, 4H, aromatic), 8.0 (s, 1H, COOH) ppm. MS-EI: for $C_{24}H_{36}N_2O_2$, calcd 384.55, found 385.30 m/z $(M+1)^+$.

4.1.19. 8-(7-Methyl-3-octylquinoxalin-2-yl)octanoic acid (3a)

Pale brown solid. Yield: 54%. R_f 0.28 (ethyl acetate/methanol; 20:80). Mp 60–62 °C. IR (KBr): 3400–2500 (b, –OH of –COOH), 3051 (–CH–, aromatic), 2922 ($-CH_2-$, asymmetric), 2852 ($-CH_2-$, symmetric), 1718 (s, –C=O of –COOH), 1620 (–C=N), 1251 ($-CH_3$), 1114 (–C–N), 1006, 887, 827, 723 and 657 cm^{-1}. MS-EI: for $C_{25}H_{38}N_2O_2$, calcd 398.58, found 400 m/z $(M+2)^+$.

4.1.20. 8-(7-Chloro-3-octylquinoxalin-2-yl)octanoic acid (3b)

Pale brown solid. Yield: 40%. R_f 0.85 (ethyl acetate/methanol; 20:80). Mp 60–67 °C; IR (KBr): 3400–2500 (b, –OH of –COOH), 3057 (–CH–, aromatic), 2922 ($-CH_2-$, asymmetric), 2852 ($-CH_2-$, symmetric), 1716 (s, –*C*=*O* of –*COOH*), 1602 (–C=N), 1564, 1471, 1309, 1172, 1082 (–C–N), 1010, 925, 831, 721 and 659 cm^{-1}. MS-EI: for $C_{24}H_{35}ClN_2O_2$, calcd 418.24, found 416 m/z $(M-2)^+$.

4.1.21. 8-(7-Nitro-3-octylquinoxalin-2-yl)octanoic acid (3c)

Pale brown solid. Yield: 52%. R_f 0.77 (ethyl acetate/methanol; 20:80). Mp 55–57 °C; IR (KBr): 3400–2500 (b, –OH of –COOH), 3093 (–CH–, aromatic), 2922 ($-CH_2-$, asymmetric), 2854 ($-CH_2-$, symmetric), 1726 (s, –*C*=*O* of –COOH), 1618 (–C=N), 1575 and 1462 ($-NO_2$), 1340, 1166, 1076 (–C–N), 1006, 954, 839, 731 and 661 cm^{-1}. MS-EI: for $C_{24}H_{35}N_3O_4$, calcd 429.26, found 429 m/z $(M)^+$.

4.1.22. 8-(3-Octylquinoxalin-2-yl)octanehydrazide (3d)

Pale yellow solid (needles). Yield: 20%. R_f 0.79 (chloroform/ethyl acetate/methanol; 80:06:14). Mp 113–115 °C. IR (KBr): 3317 (–NH), 3041 (–CH–, aromatic), 2920 ($-CH_2-$, asymmetric), 2850 ($-CH_2-$, symmetric), 1735 (*s*, –*C*=*O* of –*CONHNH*$_2$), 1602 (–C=N), 1533, 1465, 1377, 1014 (–C–N), 950, 760, 723 and 617 cm^{-1}. MS-EI: for $C_{24}H_{38}N_4O$, calcd 398.30, found 397 m/z $(M-1)^+$.

4.1.23. 8-(4-Octyl-1,2,5-oxadiazol-3-yl)octanoic acid (4)

Colourless solid. Yield: 44%. R_f 0.40 (ethyl acetate/methanol; 12:88). Mp 120–125 °C. IR (KBr): 3400–2500 (b, –OH of –COOH), 2926 ($-CH_2-$, asymmetric), 2852 ($-CH_2-$, symmetric), 1720 (*s*, –*C*=*O* of –*COOH*), 1604, 1442 (–C=N), 1188, 1033 (–C–N), 918 (–N–O) and 740 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$), δ: 0.89 (t, 3H, CH_3), 1.25–1.35 (m, 20H, $(CH_2)_n$), 1.60–1.65 (m, 4H, $(CH_2)_n$), 2.30 (m, 2H, CH_2COOH), 2.50–2.60 (m, 4H, $(CH_2)_n$), 7.50 (s, 1H, COOH) ppm: MS-EI: for $C_{18}H_{32}N_2O_3$, calcd 324.24, found 323 m/z $(M-1)^+$. MS-CI: 323 m/z $(M-1)^+$.

4.1.24. 8-(5-Octyl-3-thioxo-2,3-dihydro-1,2,4-triazin-6-yl) octanoic acid (5)

Yellow solid. Yield: 56%. R_f 0.77 (ethyl acetate/methanol; 20:80). Mp 131–136 °C; IR (KBr): 3400–2500 (b, –OH of –COOH), 2928 ($-CH_2-$, asymmetric), 2856 ($-CH_2-$, symmetric), 1739 (*s*, –*C*=*O* of –*COOH*), 1680, 1577 (–C=N), 1444, 1383, 1255 (–C=S), 1184, 1091 (–C–N), 985, 767, 725, 651 and 597 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$), δ: 0.90 (t, 3H, CH_3), 1.30–1.45 (m, 16H, $(CH_2)_n$), 1.65 (m, 4H, $(CH_2)_n$), 2.05 (m, 2H, CH_2COOH), 2.15–2.35 (m, 4H, $(CH_2)_n$), 7.8 (br s, 1H, NH), 9.05 (s, 1H, COOH) ppm. MS-EI: for $C_{19}H_{33}N_3O_2S$, calcd 367.23, found 365 m/z $(M-2)^+$.

4.1.25. 8-(3-Octyl-5,7-dioxo-6,7-dihydro-5*H*-1,4-diazepine-2-yl)octanoic acid (6)

Pale yellow solid. Yield: 60%. R_f 0.77 (ethyl acetate/methanol; 40:60). Mp 128–133 °C. IR (KBr); 3400–2500 (b, –OH of –COOH), 3365, 3175, 2924 ($-CH_2-$, asymmetric), 2852 ($-CH_2-$, symmetric), 1712 (*s*, –*C*=*O* of –*COOH*), 1650, 1591 (–C=N), 1460, 1406, 1260, 1132, 1091 (–C–N), 964, 763, 725, 688, 651 and 582 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$), δ: 0.90 (t, 3H, CH_3), 1.22–1.40 (m, 16H, $(CH_2)_n$), 1.52–1.68 (m, 4H, $(CH_2)_n$), 2.35 (m, 2H, CH_2COOH), 3.0 (d, 2H, CO–CH_2–CO), 9.50 (s, 1H, COOH) ppm. MS-EI: for $C_{21}H_{34}N_2O_4$, calcd 378.25, found 375 m/z $(M-3)^+$.

4.1.26. Methyl 8-(5-(2-acetoxyoctyl)-2,2-dimethyl-1,3-dioxolan-4-yl)octanoate (7)

Pale brownish liquid. Yield: 40%. IR (neat); 2926 ($-CH_2-$, asymmetric), 2857 ($-CH_2-$, symmetric), 1732 (s, –*C*=*O* of –*COOCH*$_3$ and –*OCOCH*$_3$), 1457, 1373, 1241, 1172, 1092, 1030, 832, 726 and 642 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$), δ: 0.90 (t, 3H, CH_3), 1.30–1.50 (m, 16H, $(CH_2)_n$), 1.65 (m, 4H, $(CH_2)_n$), 1.90-2.10 (m, 2H, CH_2COO-), 2.35 (m, 2H, CH_2), 3.35–4.00 (s, 3H, OCH_3), 4.70–5.10 (*d*, 2*H*, methylenedioxy protons) ppm. MS-EI: for $C_{22}H_{40}O_6$, calcd 400.28, found 400 m/z $(M)^+$.

4.1.27. Acetonide of 9,10-dihydroxy-methyl-12-acetylricinoleic acid (7a)

Pale orange liquid. Yield: 38%. IR (neat); 2930 ($-CH_2-$, asymmetric), 2857 ($-CH_2-$, symmetric), 1738 (*s*, –*C*=*O* of –*COOCH*$_3$

and –$OCOCH_3$), 1617, 1460, 1437, 1367, 1239, 1171, 1077, 846, 726 and 668 cm^{-1}. ^{13}C NMR (100 MHz, DMSO-d_6), δ: 13.97 (CH_3), 22.50, 23.58, 25.12, 25.48, 25.75, 25.76, 25.97, 29.22, 29.44, 29.59, 31.72, 32.96, 34.31, 35.98, 36.53, 51.32 (CH_3 of $COOCH_3$), 69.69, 80.98, 82.55, 126.44, 170.59 (CO of $OCOCH_3$) and 174.17 (CO of $COOCH_3$) ppm. MS-EI: for $C_{24}H_{44}O_6$, calcd 428.31, found 428 m/z $(M)^+$.

4.1.28. Acetonide of 9,10-dihydroxy stearic acid ethyl ester (8)

Pale orange liquid. Yield: 48%. IR (neat); 2925 (–CH_2–, asymmetric), 2857 (–CH_2–, symmetric), 1737 (s, –C=O of –$COOC_2H_5$), 1454, 1437, 1369, 1177, 1081, 974, 854 and 724 cm^{-1}.

4.2. In vitro antioxidant activity

4.2.1. DPPH method[11]

The antioxidant activities of the synthesized compounds were assessed on the basis of radical scavenging effect of the stable DPPH free radical. The assay was carried out in a 96 well microtiter plate. To 100 μl of DPPH solution, 100 μl of each of the test sample or standard solution was added separately in to microtiter plates. The final concentrations of the test solutions ranging from 10 to 0.005 mM and for standard ranging from 0.5 to 0.005 mM were used, respectively. The plates were incubated at 37 °C for 30 min and absorbance of each solution was measured at 490 nm using microtiter plate reader (ELISA) against the corresponding test and standard blank. The remaining DPPH was calculated. IC_{50} is the concentration of the sample required to scavenge, 50% DPPH free radicals.

4.3. In vitro anti-inflammatory activity

4.3.1. Inhibition of bovine serum albumin denaturation[12]

Test or Standard solutions (1 mL) containing different concentrations of drug was mixed with 1 mL of 1 mM albumin solution in phosphate buffer and incubated at 27 ± 1 °C for 15 min. Denaturation was induced by keeping the reaction mixture at 60 ± 1 °C in a water bath for 10 min. After cooling the turbidity was measured at 660 nm (UV-1660-Shimadzu Spectrophotometer). Percentage inhibition of denaturation was calculated from control where no drug was added. Each experiment was done in triplicate and average was taken.

4.4. In vivo anti-inflammatory activity

4.4.1. Acute inflammation

4.4.1.1. Carrageenan-induced paw edema in rats[13]. Acute inflammation was induced in the hind paw of rats by injecting 0.1 mL of freshly prepared 1% w/v solution of λ-carrageenan (Hi-media, India). The carrageenan was injected under the plantar region of the left hind paw and paw volumes were measured using plethysmometer (UGO Basile, Italy) at 3 h after carrageenan challenge. Increase in paw volume and percentage inhibition was calculated and expressed as amount of inflammation.

4.5. Chronic inflammation

4.5.1. Induction of chronic paw inflammation in rats[14]

Chronic inflammation was induced by intraplantar injection of 0.1 mL of 1% w/v Freund's complete adjuvant (FCA) (Calbiochem, Germany) in left hind paw of rats. Rats were divided into seven groups (n = 6). Sham control received saline intraplantarly and rest all received CFA intraplantarly. On day 21, after induction of inflammation, treatments were continued po till 35 day in respective treatment groups. Sham control and FCA control received vehicle orally while other five group received compounds **3**, **3b**, and **3c** (100 mg/kg/po) and diclofenac (5 mg/kg/po), respectively. Paw edema was measured on day 0, 21 and 35 after CFA injection using plethysmometer (UGO Basile, Italy) and expressed as amount of inflammation.

4.5.2. Myeloperoxidase assay

Myeloperoxidase activity was determined following technique reported by Graff et al.[15] The isolated segments of foot pad from different treatment groups were individually homogenized in 5 mL of phosphate buffer (0.01 M). Homogenized tissue was centrifuged at 10,000 rpm. Supernatant collected and to 50 μl supernatant/50 mM phosphate buffers (pH 6) (blank) into the respective well added 250 μl *o*-dianisidine hydrochloride (ODA) (0.167 mg/mL in 50 mM phosphate buffer, pH 6) containing 0.0005% v/v hydrogen peroxide. After 15 min incubation, added 50 μl of 4 M H_2SO_4 and took absorbance at 490 nm. The percentage of MPO activity with respect to saline control was calculated and results were expressed as mean% MPO activity ± SEM.

4.6. In vitro dipeptidylpeptidase-IV (DPP-IV) Inhibition[16]

DPP-IV activity was determined by the cleavage rate of *p*-nitro aniline (pNA) from synthetic substrate Gly-Pro-pNA. The assay was conducted by adding 25 μl mouse plasma in 100 μl of the assay buffer (25 mM Tris, pH 7.4, 140 mM NaCl, 10 mM KCl, 1% BSA) to 96-well plates. Mixture was incubated for 30 min at 37 °C after addition of 10 μl of all test compounds (500 μg/mL) and the reaction was initiated by adding 65 μl of 1000 μM substrate Gly-Pro-pNA. Conversion rate of Gly-Pro-pNA into p-nitro aniline by DPP-IV enzyme was monitored at 0 and 30 min spectrophotometrically at 405 nm using micro plate reader (Bio-Tek Instruments, USA). The experiment was performed in triplicate. Percentage inhibition was calculated at each concentration with respect to the vehicle control.

4.7. Oral glucose tolerance test (OGTT) in lean mice[17]

Animals were fasted overnight and divided into six groups (n = 6) based on basal blood glucose levels. Distilled water or glucose load of 2 g/kg/po were administered immediately after treatment with 0.5% w/v carboxymethylcellulose (CMC) or compounds (100 mg/kg) by po and ip route. Blood samples were collected by retro-orbital plexus at 0, 15, 30, 60 and 120 min after glucose challenge. Plasma glucose was measured spectrophotometrically using colorimetric kits (Aspen limited, India). The reduction in blood glucose produced by test compounds was calculated using area under the curve ($AUC_{0-120min}$). The results were expressed in plasma glucose in mg/dl and $AUC_{0-120min}$. Percentage reduction in plasma glucose was also calculated from AUC graph.

4.8. Statistical analysis

Data represent the mean ± standard error mean (SEM) of the indicated number of experiments. Graphs were prepared by GraphPad Prism 5.0 version software. Statistical analysis of the data was carried out by one-way ANOVA (GraphPad Prism 5.0 version) followed by post hoc Dunnett's test. A value of p <0.05 was considered to be significant.

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