3. MATERIALS AND METHODS

3.1 MATERIALS

3.11 Animals:

All experiments and protocols described in the present study were approved by the Institutional Animal Ethics Committee of M.S. University of Baroda and with permission from the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India. All the experimental animals were fasted overnight before the experiment.

Male Sprague Dawley rats (200±25g) were housed in group of 4 in polypropylene cage and maintained under standardized condition (12h light/dark cycle, 22±2 °C temperature) and provided free access to rat pallet food (Pranav Agro Pvt. Ltd.) and purified drinking water *ad libitium.*

Swiss Albino Mice (25±5 g) were housed in group of ten in polypropylene cage and maintained under standard condition (12h light/dark cycle, 22±2 °C temperature). They were provided free access to food pallet (Pranav Agro Pvt. Ltd.) and purified drinking water *ad libitium.*

Guinea Pig, Hartley strain (300±50g) were obtained from Sun Pharma Advanced Research Centre, Baroda, housed in group of three and maintained under standard condition(12-h light/dark cycle, 22±2 °C temperature). They were provided free access to food pallet (Pranav Agro Pvt. Ltd.) and purified drinking water *ad libitium.*

3.12 Chemicals

All Fine chemicals and reagents used in the experiments were of analytical or the highest purity grade available. Most of them were purchased from Sigma chemical company, St. Louis, U.S.A. All other general laboratory chemicals and reagents were of analytical grade and obtained from S.D. Fine chemicals Ltd. Vadodara.

Table 8. List of chemicals, instruments and drugs and their sources are given in the table below.

Chemicals / Solvents	Source
Acetylcholine	Sigma
Lipopolysaccharide	Sigma
Phenylephrine	Sigma
Carrageenan (λ grade)	Sigma
Arachidonic acid	Sigma
Lipopolysaccharide	Sigma
Acetic acid	BDH
Dimethyl sulfoxide (DMSO)	Qualigen
Nω-nitro-L-arginine-methyl ester (L-NAME)	Sigma
Epinephrine	Sigma
Mycobacterium butyricum	Sigma
Sodium chloride	Merck
Carboxy Methyl Cellulose Sodium	S D Fine
Trinitrobenzenesulfonic acid (TNBS)	Sigma
COX-1 and COX-2 colorimetric screening kit	Cayman USA
Valdecoxib	Sun Pharma
Celecoxib	Sun Pharma
Indomethacin	Sun Pharma
Aspirin	Sun Pharma

3.13 Instruments

Table 9: List of instruments used in the study.

Instruments	Model / Make	Source
Elisa Plate Reader	Molecular Device	USA
Plethysmometer	UGO Basile	Italy
Analytical Balance	Mettler Toledo	USA
Data Acquisition system	Bio-Pac	USA
Organ Bath	UGO Basile	Italy
Gemini Recorder	UGO Basile	Italy

3.14 Working solutions for various experimental protocol

(i) 1 % Carboxy Methyl Cellulose (CMC)

1% (w/v) solution of Carboxy methyl cellulose (CMC) was prepared by solving 1 gm of CMC in 100 ml of distilled water.

(ii) 1% Acetic Acid

1% (v/v) of acetic acid solution was prepared by diluting 0.1ml of acetic acid in 9.9 ml normal saline. Stirred for 15-20 minutes. 10 ml/kg of acetic acid was used to induce writhing response in mice.

(iii) 1% Carrageenan

100 mg of Carrageenan was dissolved in 10 ml of sterile normal saline to make 1% (w/v) solution. 0.1 ml of dissolved carrageenan was used to induce rat paw edema.

(iv) Test Drugs:

1. NCE: MCR Series compounds solutions

   For *In vitro* experiments- All Synthetic MCR series compounds were dissolved into DMS0 e.g. 25mg compound dissolved in 0.25ml and then serially diluted with phosphate buffer saline pH-7.4(PBS).

   For *In vivo* experiments- 25mg of compounds dissolved in 0.25 ml DMSO and resuspended in 10 ml of 1% CMC.

2. Herbal Drug extract.

   For *In vitro* experiments- All herbal drug extracts were dissolved in to 100% methanol first to make stock solution and then further diluted.

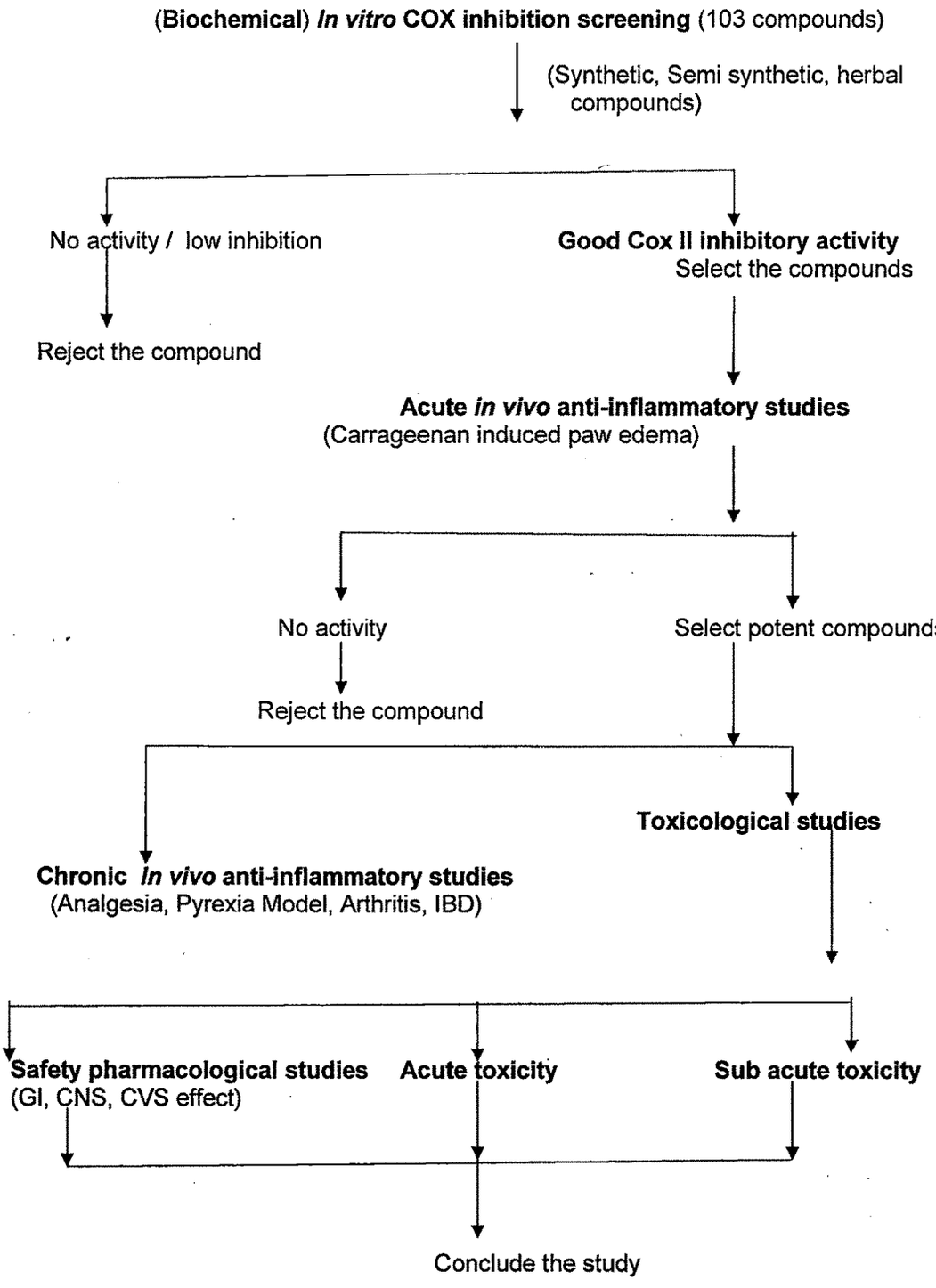
   For *In vivo* studies-500mg of compounds dissolved in 0.25ml ethanol and resuspended in 10ml of 1%CMC.

(V) Reference Standards - e.g. Celecoxib, Rofecoxib, Indomethacin

For *In vitro* experiments- All Standard drugs were dissolved into DMS0 e.g. 25mg compound dissolved in 0.25ml and then serially diluted in phosphate buffer saline pH-7.4(PBS).

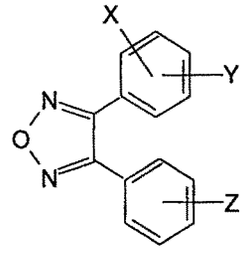
For *In vivo* experiments- 25mg of compounds dissolved in 0.25ml DMSO and resuspended in 10ml of 1% CMC

3.15 Methodology – compound screening –flow chart

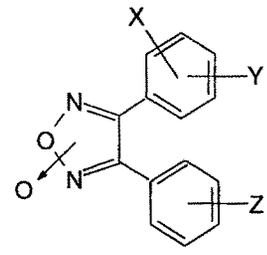


3.16 Test Compounds

3.16. a. Synthesized Compounds



(I)

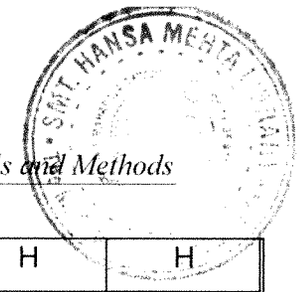


(II)

Compounds	MCR no.	X	y	z
3,4-Diphenyl-1,2,5-oxadiazole	MCR-49	H	H	H
3,4-Di [4-anisyl]-1,2,5-oxadiazole	MCR-50	p-OMe	H	p-OMe
3-[4-Anisyl]-4-[2-chlorophenyl]-1,2,5-oxadiazole	MCR-127	p-OMe	H	o-Cl
3-[4-Dimethylaminophenyl]-4-[2-chlorophenyl]-1,2,5-oxadiazole	MCR-102	p-N(Me_2)	H	o-Cl
3-[3,4-Dimethoxyphenyl]-4-[2-chlorophenyl]-1,2,5-oxadiazole	MCR-166	p-OMe	m-OMe	o-Cl
3-[4-Anisyl]-4-phenyl-1,2,5-oxadiazole	MCR-167	p-OMe	H	H
3-[4-Tolyl]-4-phenyl-1,2,5-oxadiazole	MCR-95	p-Me	H	H
3-[4-Chlorophenyl]-4-phenyl-1,2,5-oxadiazole	MCR-101	p-Cl	H	H
3-[4-Flourophenyl]-4-phenyl-1,2,5-oxadiazole	MCR-161	p-F	H	H
3-[4-Bromophenyl]-4-phenyl-1,2,5-oxadiazole	MCR-163	p-Br	H	H
3-[4-Nitrophenyl]-4-phenyl-1,2,5-oxadiazole	MCR-169	p-NO_2	H	H
3-[4-Chlorophenyl]-4-[4-tolyl] -1,2,5-oxadiazole	MCR-165	p-Cl	H	p-Me

3-[4-Chlorophenyl]-4-[4-anisyl] -1,2,5-oxadiazole	MCR-162	p-Cl	H	p-OMe
3-[4-Chlorophenyl]-4-[4-fluorophenyl]-1,2,5-oxadiazole	MCR-164	p-Cl	H	p-F
3-[4-Methanethiophenyl]-4-phenyl-1,2,5-oxadiazole	MCR-125	p-SMe	H	H
3-[4-Methanesulphonylphenyl]-4-phenyl-1,2,5-oxadiazole	MCR-165	p-SO_2Me	H	H
3-[4-Methanesulphonylphenyl]-4-[4-tolyl-1,2,5-oxadiazole	MCR-162	p-SO_2Me	H	p-Me
3-[4-Methanesulphonylphenyl]-4-[4-flourophenyl]-phenyl-1,2,5-oxadiazole	MCR-164	p-SO_2Me	H	p-F
3,4-Di [4-Nitrophenyl]-1,2,5-oxadiazole	MCR-125	p-NO_2	H	p-NO_2
3-[3-Nitrophenyl]-4-[4-nitrophenyl-1,2,5-oxadiazole	MCR-168	H	m-NO_2	p-NO_2
3,4-Di [4-sulphamoylphenyl]-1,2,5-oxadiazole	MCR-172	p-SO_2NH_2	H	p-SO_2NH_2
3-[4-Sulphamoylphenyl]-4-phenyl-1,2,5-oxadiazole	MCR-173	p-SO_2NH_2	H	H
3-(1-biphenyl)-4-phenyl-1,2,5-oxadiazole	MCR-178	p-C6H5	H	H
3-(1-biphenyl)-4-(4-tolyl)-1,2,5-oxadiazole	MCR-174	p-C6H5	H	p-CH3
3,4-Diphenyl-1,2,5-oxadiazole-N-oxide	MCR-165	H	H	H
3,4-Di [4-anisyl]-1,2,5-oxadiazole-N-oxide	MCR-207	p-OMe	H	p-NO_2
3-[4-Anisyl]-4-[2-chlorophenyl]-1,2,5-oxadiazole-N-oxide	MCR-218	p-OMe	H	o-Cl
3-[3,4-Dimethoxyphenyl]-4-[2-chlorophenyl]- 1,2,5-oxadiazole-N-oxide	MCR-219	p-OMe	m-OMe	o-Cl
3-[4-Anisyl]-4-phenyl-1,2,5-oxadiazole-N-oxide	MCR-289	p-OMe	H	H

3-[4-Tolyl]-4-phenyl-1,2,5-oxadiazole-N-oxide	MCR-290	p-Me	H	H
3-[4-Chlorophenyl]-4-phenyl-1,2,5-oxadiazole- N-oxide	MCR-285	p-Cl	H	H
3-[4-Flourophenyl]-4-phenyl-1,2,5-oxadiazole- N-oxide	MCR-175	p-F	H	H
3-[4-Chlorophenyl]-4-[4-tolyl]-1,2,5-oxadiazole- N-oxide	MCR-176	p-Cl	H	p-Me
3-[4-Chlorophenyl]-4-[4-anisyl]-1,2,5-oxadiazole-N-oxide	MCR-177	p-Cl	H	p-OMe
3-[4-Chlorophenyl]-4-[4-fluorophenyl]-1,2,5-oxadiazole-N-oxide	MCR-206	p-Cl	H	p-F
3-[4-Methanesulphonylphenyl]-4-phenyl-1,2,5-oxadiazole-N-oxide	MCR-207	-OCH3	-SO2CH3	H
3,4-Diphenyl-1,2,5-oxadiazole	MCR-217	H	H	H
3,4-Di [4-anisyl]-1,2,5-oxadiazole	MCR-218	p-OMe	H	p-OMe
3-[4-Anisyl]-4-[2-chlorophenyl]-1,2,5-oxadiazole	MCR-219	p-OMe	H	o-Cl
3-[4-Dimethylaminophenyl]-4-[2-chlorophenyl]-1,2,5-oxadiazole	MCR-220	p-N(Me2)	H	o-Cl
3-[3,4-Dimethoxyphenyl]-4-[2-chlorophenyl]-1,2,5-oxadiazole	MCR-221	p-OMe	m-OMe	o-Cl
3-[4-Anisyl]-4-phenyl-1,2,5-oxadiazole	MCR-230	p-OMe	H	H
3-[4-Tolyl]-4-phenyl-1,2,5-oxadiazole	MCR-231	p-Me	-SO2CH3	H
3-[4-Chlorophenyl]-4-phenyl-1,2,5-oxadiazole	MCR-222	p-Cl	H	H
3-[4-Flourophenyl]-4-phenyl-1,2,5-oxadiazole	MCR-232	p-F	H	p-F
3-[4-Bromophenyl]-4-phenyl-1,2,5-oxadiazole	MCR-233	p-Br	H	H

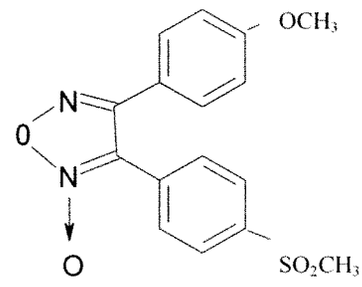


3-[4-Nitrophenyl]-4-phenyl-1,2,5-oxadiazole	MCR-234	p-NO2	H	H
3-[4-Chlorophenyl]-4-[4-tolyl] -1,2,5-oxadiazole	MCR-223	p-Cl	H	p-Me
3-[4-Chlorophenyl]-4-[4-anisyl] -1,2,5-oxadiazole	MCR-235	p-Cl	H	p-OMe
3-[4-Chlorophenyl]-4-[4-fluorophenyl]-1,2,5-oxadiazole	MCR-240	p-Cl	H	p-F
3-[4-Methanethiophenyl]-4-phenyl-1,2,5-oxadiazole	MCR-241	p-SMe	H	H
3-[4-Methanesulphonylphenyl]-4-phenyl-1,2,5-oxadiazole	MCR-243	p-SO2Me	H	H
3-[4-Methanesulphonylphenyl]-4-[4-tolyl-1,2,5-oxadiazole	MCR-242	p-SO2Me	H	p-Me
3-[4-Methanesulphonylphenyl]-4-[4-flourophenyl]-phenyl-1,2,5-oxadiazole	MCR-262	p-SO2Me	H	p-F
3,4-Di [4-Nitrophenyl]-1,2,5-oxadiazole	MCR-263	p-NO2	H	p-NO2
3-[3-Nitrophenyl]-4-[4-nitrophenyl-1,2,5-oxadiazole	MCR-264	H	m-NO2	p-NO2
3,4-Di [4-sulphamoylphenyl]-1,2,5-oxadiazole	MCR-265	p-SO2NH2	H	p-SO2NH2
3-[4-Sulphamoylphenyl]-4-phenyl-1,2,5-oxadiazole	MCR-273	p-SO2NH2	H	H
3-(1-biphenyl)-4-phenyl-1,2,5-oxadiazole	MCR-267	p-C6H5	H	H
3-(1-biphenyl)-4-(4-tolyl)-1,2,5-oxadiazole	MCR-268	p-C6H5	H	p-CH3
3,4-Diphenyl-1,2,5-oxadiazole-N-oxide	MCR-270	H	H	H
3,4-Di [4-anisyl]-1,2,5-oxadiazole-N-oxide	MCR-271	p-OMe	H	p-OMe
3-[4-Anisyl]-4-[2-chlorophenyl]-1,2,5-oxadiazole-N-oxide	MCR-289	p-OMe	H	o-Cl
3-[3,4-Dimethoxyphenyl]-4-[2-chlorophenyl]- 1,2,5-oxadiazole-N-oxide	MCR-290	p-OMe	m-OMe	o-Cl

3-[4-Anisyl]-4-phenyl-1,2,5-oxadiazole-N-oxide	MCR-285	p-OMe	H	H
3-[4-Tolyl]-4-phenyl-1,2,5-oxadiazole-N-oxide	MCR-286	p-Me	H	H
3-[4-Chlorophenyl]-4-phenyl-1,2,5-oxadiazole- N-oxide	MCR-287	p-Cl	H	H
3-[4-Flourophenyl]-4-phenyl-1,2,5-oxadiazole- N-oxide	MCR-291	p-F	H	H
3-[4-Chlorophenyl]-4-[4-tolyl]-1,2,5-oxadiazole- N-oxide	MCR-293	p-Cl	H	p-Me
3-[4-Chlorophenyl]-4-[4-anisyl]-1,2,5-oxadiazole-N-oxide	MCR-292	p-Cl	H	p-OMe
3-[4-Chlorophenyl]-4-[4-fluorophenyl]-1,2,5-oxadiazole-N-oxide	MCR-295	p-Cl	H	p-F
3-[4-Methanesulphonylphenyl]-4-phenyl-1,2,5-oxadiazole-N-oxide	MCR-296	-OCH3	-SO2CH3	H
3,4-Diphenyl-1,2,5-oxadiazole	MCR-297	H	H	H
3,4-Di [4-anisyl]-1,2,5-oxadiazole	MCR-301	p-OMe	H	p-OMe
3-[4-Anisyl]-4-[2-chlorophenyl]-1,2,5-oxadiazole	MCR-302	p-OMe	H	o-Cl
3-[4-Dimethylaminophenyl]-4-[2-chlorophenyl]-1,2,5-oxadiazole	MCR-303	p-N(Me2)	H	o-Cl
3-[3,4-Dimethoxyphenyl]-4-[2-chlorophenyl]-1,2,5-oxadiazole	MCR-313	p-OMe	m-OMe	o-Cl
3-[4-Anisyl]-4-phenyl-1,2,5-oxadiazole	MCR-314	p-OMe	H	H
3-[4-Tolyl]-4-phenyl-1,2,5-oxadiazole	MCR-315	p-Me	H	H
3-[4-Chlorophenyl]-4-phenyl-1,2,5-oxadiazole	MCR-316	p-Cl	H	H

3-[4-Flourophenyl]-4-phenyl-1,2,5-oxadiazole	MCR-317	p-F	H	H
3-[4-Bromophenyl]-4-phenyl-1,2,5-oxadiazole	MCR-318	p-Br	H	H
3-[4-Nitrophenyl]-4-phenyl-1,2,5-oxadiazole	MCR-319	p-NO2	H	H
3-[4-Chlorophenyl]-4-[4-tolyl] -1,2,5-oxadiazole	MCR-320	p-Cl	H	p-Me
3-[4-Chlorophenyl]-4-[4-anisyl] -1,2,5-oxadiazole	MCR-321	p-Cl	H	p-OMe
3-[4-Chlorophenyl]-4-[4-fluorophenyl]-1,2,5-oxadiazole	MCR-322	$-NH_2$	-OCH3	p-F
3-[4-Methanethiophenyl]-4-phenyl-1,2,5-oxadiazole	MCR-323	p-SMe	H	H
3-[4-Methanesulphonylphenyl]-4-phenyl-1,2,5-oxadiazole	MCR-324	p-SO2Me	H	H
3-[4-Methanesulphonylphenyl]-4-[4-tolyl-1,2,5-oxadiazole	MCR-326	p-SO2Me	H	p-Me
3-[4-Methanesulphonylphenyl]-4-[4-flourophenyl]-phenyl-1,2,5-oxadiazole	MCR-327	p-SO2Me	H	p-F
3,4-Di [4-Nitrophenyl]-1,2,5-oxadiazole	MCR-328	p-NO2	H	p-NO2
3-[3-Nitrophenyl]-4-[4-nitrophenyl-1,2,5-oxadiazole	MCR-329	H	m-NO2	p-NO2
3,4-Di [4-sulphamoylphenyl]-1,2,5-oxadiazole	MCR-304	p-SO2NH2	H	p-SO2NH2
3-[4-Sulphamoylphenyl]-4-phenyl-1,2,5-oxadiazole	MCR-305	p-SO2NH2	H	H
3-(1-biphenyl)-4-phenyl-1,2,5-oxadiazole	MCR-306	p-C6H5	H	H
3-(1-biphenyl)-4-(4-tolyl)-1,2,5-oxadiazole	MCR-307	p-C6H5	H	p-CH3
3,4-Diphenyl-1,2,5-oxadiazole-N-oxide	MCR-308	H	p-OMe	H

3,4-Di [4-anisyl]-1,2,5-oxadiazole-N-oxide	MCR-333	p-OMe	o-Cl	p-OMe
3-[4-Anisyl]-4-[2-chlorophenyl]-1,2,5-oxadiazole-N-oxide	MCR-335	p-OMe	o-Cl	o-Cl
3-[3,4-Dimethoxyphenyl]-4-[2-dichlorophenyl]- 1,2,5-oxadiazole-N-oxide	MCR-336	p-OMe	o-Cl	o-Cl
3-[4-Anisyl]-4-phenyl-1,2,5-oxadiazole-N-oxide	MCR-334	p-OMe	H	H
3-[4-Tolyl]-4-phenyl-1,2,5-oxadiazole-N-oxide	MCR-344	p-Me	H	H
3-[4-Chlorophenyl]-4-phenyl-1,2,5-oxadiazole- N-oxide	MCR-345	p-Cl	H	H
3-[4-Flourophenyl]-4-phenyl-1,2,5-oxadiazole- N-oxide	MCR-349	p-F	H	H
3-[4-Chlorophenyl]-4-[4-tolyl]-1,2,5-oxadiazole- N-oxide	MCR-352	p-Cl	H	p-Me
3-[4-Chlorophenyl]-4-[4-anisyl]-1,2,5-oxadiazole-N-oxide	MCR-362	p-Cl	H	p-OMe
3-[4-Chlorophenyl]-4-[4-fluorophenyl]-1,2,5-oxadiazole-N-oxide	MCR-364	p-Cl	p-Me	p-F
3-[4-Methanesulphonylphenyl]-4-phenyl-1,2,5-oxadiazole-N-oxide	MCR-363	-OCH3	-SO2CH3	H



Chemical structure - MCR-363
(3-[4-Methanesulphonylphenyl]-4-phenyl 1,2,5-oxadiazole N-oxide)

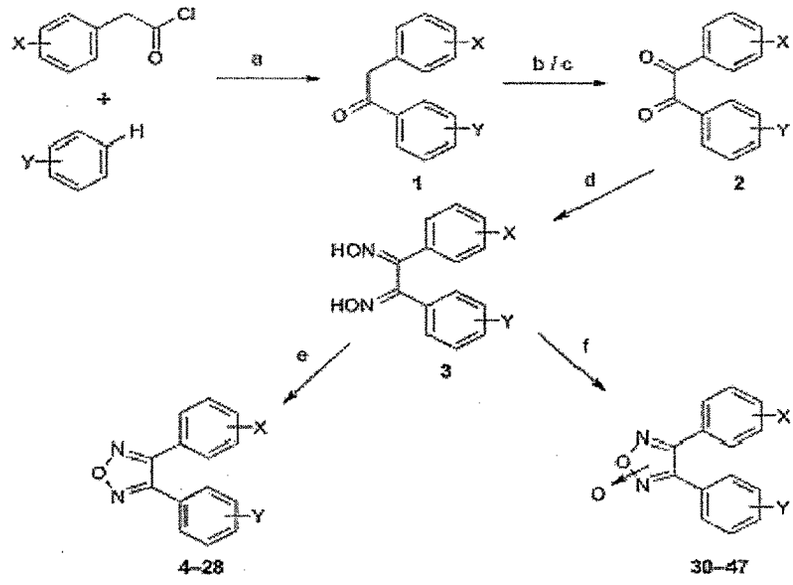
3.16 b. Synthetic pathways for compounds-

Synthetic pathway is presented below in Schemes 1 and 2, and physicochemical and spectral data for the synthesized compounds are given in Tables I and II. The starting compounds, 1,2-diaryl-1,2-ethanediones (benzils), were prepared by two routes. The first route involved benzoin condensations followed by oxidation while the second involved Friedel-Crafts acylation followed by selenium dioxide oxidation.

Syntheses of 1,2-diaryl-1,2-ethanedione dioximes. – A mixture of 1,2-diaryl-1,2-ethanediones (benzils) (10 mmol), hydroxylamine hydrochloride (60 mmol) and pyridine (10 mL) was refluxed on an oil bath for 7 h. The reaction mixture was poured onto crushed ice containing concentrated hydrochloric acid (10 mL). The precipitate obtained was filtered, washed with cold water and dried. The crude materials were used as such for the next step without further purification.

Syntheses of 3,4-diaryl-1,2,5-oxadiazoles. – A mixture of 1,2-diaryl- 1,2-ethanedione dioximes (4 mmol) and succinic anhydride (20 mmol) was heated at 180–185 °C for 10 min in an oil-bath. The molten product was cooled, suspended in water and a sufficient quantity of sodium bicarbonate was added to neutralize the acid. The resulting mixture was extracted with successive quantities of chloroform. The combined organic extract was washed with water, dried and the

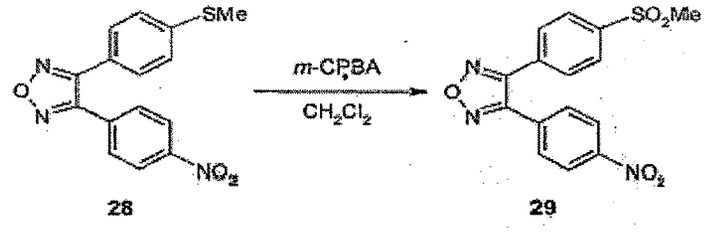
solvent was recovered. The product obtained was crystallized from methanol to yield the title compounds.



X = H, 2-Cl, 4-Cl, 4-F, 4-Me, 4-OMe, NO_2
Y = H, 4-Cl, 4-Br, 4-F, 4-Me, 4-OMe, 4-SMe, 4-SO_2Me, 3,4-di-OMe

Reagents and conditions: a – $AlCl_3$, CH_2Cl_2, 0–60 °C, 3–4 h; b – SeO_2, Ac_2O, reflux, 1–8 h; c – SeO_2, DMSO, microwave irradiation, 30–90 s; d – $NH_2OH \times HCl$, C_6H_5N, reflux, 7–8 h; e – $(-CH_2CO)_2O$, 80–5 °C, 10 min; f – aq. NaOCl (20%), 5–20 °C, 14–16 h

Scheme 1



Scheme 2

Characterization data of 3,4-diaryl-1,2,5-oxadiazoles (4–29)

Compd. No.	X	Y	M.p. (°C)	Yield (%)	Molecular formula (M_r) and mass (*m/z*)	Elemental analysis, found/calcd. (%) C	H	N	IR (ν, cm^{-1})	^1H NMR (δ, ppm)
4	H	H	93–96[a]	49	$C_{14}H_{10}N_2O$ (222.25) 223 (M+H)	–	–	–	1577 (C=N-O), 1442 (N-O)	7.56–7.37 (m, 10H, ArH)
5	H	4'-Cl	83–84	21	$C_{14}H_9ClN_2O$ (256.69) 256 (M^+)	65.38 65.51	3.74 3.53	10.67 10.91	1597 (C=N-O), 1447 (N-O)	7.62–7.35 (m, 9H, ArH)
6	H	4'-Br	111–113	28	$C_{14}H_9BrN_2O$ (301.14) 302 (M+H)	56.12 55.84	2.79 3.01	9.48 9.30	1596 (C=N-O), 1446 (N-O)	7.59–7.39 (m, 9H, ArH)
7	H	4'-F	85–87	33	$C_{14}H_9FN_2O$ (240.24) 240 (M^+)	69.88 70.00	3.53 3.78	11.87 11.66	1605 (C=N-O), 1454 (N-O)	7.56–7.40 (m, 7H, ArH), 7.15–7.09 (m, 2H, 3',5'-ArH)
8	H	4'-Me	57–59	30	$C_{15}H_{12}N_2O$ (236.28) 236 (M^+)	75.96 76.25	4.87 5.12	11.59 11.86	1605 (C=N-O), 1450 (N-O)	7.54–7.20 (m, 9H, ArH), 2.40 (s, 3H, $ArCH_3$)
9	H	4'-OMe	67–69	36	$C_{15}H_{12}N_2O_2$ (252.28) 252 (M^+)	71.77 71.42	4.64 4.79	10.97 11.10	1613 (C=N-O), 1456 (N-O), 1250 (Ar-O-Me, asym), 1025 (Ar-O-Me, sym)	7.55–7.37 (m, 7H, ArH), 6.91–6.88 (m, 2H, 3',5'-ArH), 3.82 (s, 3H, $ArOCH_3$)
10	H	4'-SMe	83–84	27	$C_{15}H_{12}N_2OS$ (268.34) 269 (M+H)	67.47 67.14	4.35 4.51	10.62 10.44	1602 (C=N-O), 1435 (N-O)	7.54–7.40 (m, 7H, ArH), 7.26–7.23 (m, 2H, 3',5'-ArH), 2.50 (s, 3H, $ArSCH_3$)
11	H	4'-SO_2Me	142–143[b]	47	$C_{15}H_{12}N_2O_3S$ (300.34)	60.26 59.99	4.32 4.03	9.16 9.33	1600 (C=N-O), 1448 (N-O), 1308 (SO_2 asym), 1149 (SO_2 sym)	8.03–8.00 (m, 2H, 3',5'-ArH), 7.78–7.75 (m, 2H, 2',6'ArH), 7.57–7.44 (m, 5H, ArH), 3.11 (s, 3H, $ArSO_2CH_3$)

Table Continued

Compd. No.	X	Y	M.p. (°C)	Yield (%)	Molecular formula (M_r) and mass (*m/z*)	Elemental analysis, found/calcd. (%) C	H	N	IR (ν, cm^{-1})	^1H NMR (δ, ppm)
12	2-Cl	H	59–60	26	$C_{14}H_9ClN_2O$ (256.69)	65.06 65.51	4.87 4.53	10.74 10.94	1600 (C=N-O), 1434 (N-O)	7.51–7.31 (m, 9H, ArH)
13	2-Cl	4'-Me	117–118	38	$C_{15}H_{11}ClN_2O$ (270.72)	66.90 66.55	3.79 4.10	10.55 10.35	1613 (C=N-O), 1435 (N-O)	7.51–7.39 (m, 4H, ArH), 7.37–7.34 (m, 2H, 2',6'-ArH), 7.16–7.13 (m, 2H, 3',5'-ArH), 2.35 (s, 3H, $ArCH_3$)
14	2-Cl	4'-OMe	107–108	39	$C_{15}H_{11}ClN_2O_2$ (286.72) 287 (M^+)	62.48 62.84	3.50 3.87	9.99 9.77	1611 (C=N-O), 1437 (N-O), 1253 (Ar-O-Me, asym), 1029 (Ar-O-Me, sym)	7.53–7.39 (m, 6H, ArH), 6.88–6.83 (m, 2H, 3',5'-ArH), 3.80 (s, 3H, $ArOCH_3$)
15	2-Cl	3',4'-di-OMe	77–79	60	$C_{16}H_{13}ClN_2O_3$ (316.75) 316 (M^+)	60.48 60.67	3.86 4.14	9.12 8.84	1605 (C=N-O), 1440 (N-O), 1256 (Ar-O-Me, asym), 1019 (Ar-O-Me, sym)	7.52–7.43 (m, 4H, ArH), 7.08–7.087 (dd, 1H, 2'-ArH), 7.02–6.98 (dd, 1H, 6'-ArH), 6.81–6.78 (dd, 1H, 3'-ArH), 3.87 (s, 3H, $ArOCH_3$), 3.71 (s, 3H, $ArOCH_3$)
16	4-Cl	4'-F	101–103	27	$C_{14}H_8FClN_2O$ (274.68)	60.82 61.22	3.22 2.94	10.38 10.20	1601 (C=N-O), 1447 (N-O)	7.54–7.48 (m, 2H, 2',6'-ArH), 7.46–7.41 (m, 4H, ArH), 7.19–7.11 (m, 2H, 3',5'-ArH)
17	4-Cl	4'-Me	137–139	19	$C_{15}H_{11}ClN_2O$ (270.72)	66.91 66.55	4.26 4.10	10.53 10.35	1602 (C=N-O), 1445 (N-O)	7.50–7.41 (m, 4H, ArH), 7.37–7.34 (m, 2H, 2',6'-ArH), 7.16–7.13 (m, 2H, 3',5'-ArH), 2.46 (s, 3H, $ArCH_3$)
18	4-Cl	4'-OMe	98–100	26	$C_{15}H_{11}ClN_2O_2$ (286.72)	62.57 62.84	4.28 3.87	9.95 9.77	1614 (C=N-O), 1451 (N-O), 1253 (Ar-O-Me, asym), 1027 (Ar-O-Me, sym)	7.51–7.40 (m, 6H, ArH), 6.98–6.93 (m, 2H, 3',5'-ArH), 3.86 (s, 3H, $ArOCH_3$)

Table Continued

Compd. No.	X	Y	M.p. (°C)	Yield (%)	Molecular formula (M_r) and mass (m/z)	Elemental analysis, found/calcd. (%)			IR (ν, cm^{-1})	^1H NMR (δ, ppm)
						C	H	N		
19	4-F	4'-SO_2Me	155–157	32	$C_{15}H_{11}FN_2O_3S$ (318.33)	56.33 56.60	3.86 3.48	8.56 8.80	1606 (C=N-O), 1310 (SO_2 asym), 1151 (SO_2 sym)	8.05–8.02 (m, 2H, 3',5'-ArH), 7.77–7.74 (m, 2H, 2',6'ArH), 7.53–7.49 (m, 2H, 2,6-ArH), 7.20–7.14 (m, 2H, 3,5-ArH), 3.12 (s, 3H, $ArSO_2CH_3$)
20	4-Me	4'-SO_2Me	141–142	43	$C_{16}H_{14}N_2O_3S$ (314.37)	61.48 61.13	4.24 4.49	9.02 8.91	1610 (C=N-O), 1451 (N-O), 1307 (SO_2 asym),1150 (SO_2 sym)	8.03–8.00 (m, 2H, 3',5'-ArH), 7.78–7.75 (m, 2H, 2',6'-ArH), 7.39–7.35 (m, 2H, 2,6-ArH), 7.28–7.25 (m, 2H, 3,5-ArH), 3.10 (s, 3H, $ArSO_2CH_3$), 2.43 (s, 3H, $ArCH_3$)
21	4-OMe	4'-OMe	123–125	26	$C_{16}H_{14}N_2O_3$ (282.30) 283 (M+H)	67.83 68.08	4.66 5.00	10.16 9.92	1612 (C=N-O), 1444 (N-O), 1257 (Ar-O-Me, asym), 1026 (Ar-O-Me, sym)	7.53–7.45 (m, 4H, ArH), 6.97–6.92 (m, 4H, 3,3',5,5'-ArH), 3.86 (s, 6H, $ArOCH_3$)
22	4-NO_2	H	139–142	22	$C_{14}H_9N_3O_3$ (267.25) 267 (M^+)	63.24 62.92	3.02 3.39	15.58 15.72	1602 (C=N-O), 1515 (NO_2, asym), 1448 (N–O), 1350 (NO_2, sym)	8.31–8.28 (m, 2H, 3,5-ArH), 7.78–7.74 (m, 2H, 2,6-ArH), 7.58–7.45 (m, 5H, ArH)
23	4-NO_2	4'-Cl	168–170	53	$C_{14}H_8ClN_3O_3$ (301.69)	55.52 55.74	2.98 2.67	13.77 13.93	1601 (C=N-O), 1515 (NO_2, asym), 1448 (N–O) and 1350 (NO_2, sym)	8.34–8.31 (m, 2H, 3,5-ArH), 7.77–7.73 (m, 2H, 2,6-ArH), 7.50–7.43 (m, 4H, ArH)
24	4-NO_2	4'-Br	185–186	30	$C_{14}H_8BrN_3O_3$ (346.14)	48.16 48.58	2.66 2.33	12.38 12.14	1600 (C=N-O), 1517 (NO_2, asym), 1442 (N-O) and 1346 cm^{-1} (NO_2, sym)	8.34–8.30 (m, 2H, 3,5-ArH), 7.77–7.72 (m, 2H, 2,6-ArH), 7.65–7.60 (m, 2H, 2',6'- ArH), 7.40–7.36 (m, 2H, 3',5'-ArH)

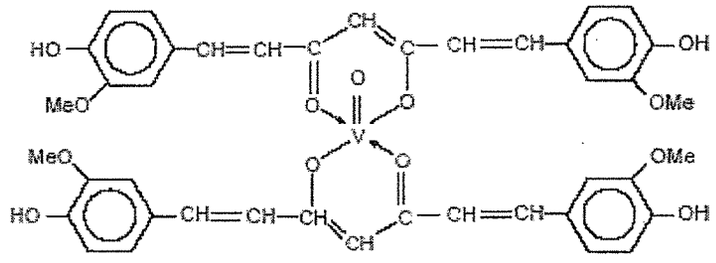
Table – Characterization data of 3,4-diaryl-1,2,5-oxadiazole N-oxides (30–47)

Compd. No.	X	Y	M.p. (°C)	Yield (%)	Molecular formula (M_r) and mass (*m/z*)	Elemental analysis, found/calcd. (%)			IR (ν, cm^{-1})	^1H NMR (δ, ppm)
						C	H	N		
30	H	H	114–117[a]	65	$C_{14}H_{10}N_2O_2$ (238.25)	–	–	–	1592 (C=N⁺-O⁻), 1419 (=N⁺(O⁻)-O)	7.55–7.35 (m, 10H, ArH)
31	H	4′-Cl	104–105	33	$C_{14}H_9ClN_2O_2$ (272.69)	61.35 61.66	3.59 3.33	10.54 10.27	1591 (C=N⁺-O⁻), 1434 (=N⁺(O⁻)-O)	7.56–7.39 (m, 9H, ArH)
32	H	4′-F	113–115	30	$C_{14}H_9FN_2O_2$ (256.24) 256 (M^+)	65.47 65.62	3.79 3.54	11.12 10.93	1588 (C=N⁺-O⁻), 1429 (=N⁺(O⁻)-O)	7.56–7.40 (m, 7H, ArH), 7.17–7.08 (m, 2H, 3′,5′-ArH)
33	H	4′-Me	104–106	53	$C_{15}H_{12}N_2O_2$ (252.28) 252 (M^+)	71.09 71.42	4.48 4.79	11.35 11.10	1600 (C=N⁺-O⁻), 1429 (=N⁺(O⁻)-O)	7.54–7.38 (m, 7H, ArH), 7.25–7.18 (m, 2H, 3′,5′-ArH), 2.42 and 2.40 (s, 3H, $ArCH_3$)
34	H	4′-OMe	103–105	49	$C_{15}H_{12}N_2O_3$ (268.27)	67.42 67.16	4.82 4.51	10.57 10.44	1591 (C=N⁺-O⁻), 1425 (=N⁺(O⁻)-O), 1252(Ar-O-Me, asym), 1026 (Ar-O-Me, sym)	7.54–7.40 (m, 7H, ArH), 6.94–6.89 (m, 2H, 3′,5′-ArH), 3.84 and 3.83 (s, 3H, $ArOCH_3$)
35	H	4′-SO_2Me	125–127[b]	35	$C_{15}H_{12}N_2O_4$ (316.34) 316 (M^+)	57.33 56.95	3.67 3.82	8.67 8.86	1594 (C=N⁺-O⁻), 1448 (=N⁺(O⁻)-O), 1307 (SO_2 asym), 1150 cm^{-1} (SO_2 sym)	8.03–8.00 (m, 2H, 3′,5′-ArH), 7.79–7.74 (m, 2H, 2′,6′-ArH), 7.58–7.44 (m, 5H, ArH), 3.11 and 3.09 (s, 3H, $ArSO_2CH_3$)
36	2-Cl	4′–Me	139–140	66	$C_{15}H_{11}ClN_2O_2$ (286.72)	62.66 62.84	3.47 3.87	9.52 9.77	1592 (C=N⁺-O⁻), 1423 (=N⁺(O⁻)-O)	7.56–7.11 (m, 8H, ArH), 2.36 and 2.34 (s, 3H, $ArCH_3$)
37	2-Cl	4′-OMe	114–117	53	$C_{15}H_{11}ClN_2O_3$ (302.72)	59.91 59.52	3.82 3.66	9.07 9.25	1590 (C=N⁺-O⁻), 1426 (=N⁺(O⁻)-O), 1253 (Ar-O-Me, asym), 1029 (Ar-O-Me, sym)	7.57–7.37 (m, 6H, ArH), 6.88–6.84 (m, 2H, 3′,5′-ArH), 3.81 and 3.80 (s, 3H, $ArOCH_3$)

Compd. No.	X	Y	M.p. (°C)	Yield (%)	Molecular formula (M_r) and mass (m/z)	Elemental analysis, found/calcd. (%) C	H	N	IR (ν, cm^{-1})	^1H NMR (δ, ppm)
38	2-Cl	3′,4′-di-OMe	110–111	35	$C_{16}H_{13}ClN_2O4$ (332.75)	57.51 57.76	3.62 3.94	8.28 8.40	1601 (C=N^+-O^-), 1438 (=N^+(O^-)-O), 1264 (Ar-O-Me, asym), 1025 (Ar-O-Me, sym)	7.59–7.42 (m, 4H, ArH), 7.17–7.14 (m, 1H, 2′-ArH), 7.07–7.04 (m, 1H, 6′-ArH), 6.84–6.78 (m, 1H, 3′-ArH), 3.87, 3.72 and 3.63 (s, 6H, $ArOCH_3$)
39	4-Cl	4′-F	113–115	52	$C_{14}H_8ClFN_2O_2$ (290.68) 290 (M^+)	57.41 57.85	3.02 2.77	9.82 9.64	1605 (C=N^+-O^-), 1440 (=N^+(O^-)-O)	7.54–7.41 (m, 6H, ArH), 7.19–7.13 (m, 2H, 3′,5′-ArH)
40	4-Cl	4′-Me	121–123	37	$C_{15}H_{11}ClN_2O_2$ (286.72) 286 (M^+)	62.56 62.84	3.59 3.87	9.59 9.77	1589 (C=N^+-O^-), 1436 (=N^+(O^-)-O)	7.50–7.37 (m, 6H, ArH), 7.27–7.24 (m, 2H, 3′,5′-ArH), 2.42 and 2.40 (s, 3H, $ArCH_3$)
41	4-Cl	4′-OMe	142–144	53	$C_{15}H_{11}ClN_2O_3$ (302.72)	59.88 59.52	3.87 3.66	9.42 9.25	1591 (C=N^+-O^-), 1450 (=N^+(O^-)-O), 1254 (Ar-O-Me, asym), 1025 (Ar-O-Me, sym)	7.51–7.40 (m, 6H, ArH), 6.97–6.94 (m, 2H, 3′,5′-ArH), 3.86 and 3.85 (s, 3H, $ArOCH_3$)
42	4-OMe	4′-OMe	110–114	56	$C_{16}H_{14}N_2O_4$ (298.30) 299 (M+H)	64.66 64.42	4.99 4.73	9.17 9.39	1600 (C=N^+-O^-), 1443 (=N^+(O^-)-O)1261 (Ar-O-Me, asym), 1022 (Ar-O-Me, sym)	7.50–7.43 (m, 4H, ArH), 6.97–6.92 (m, 4H, 3′,5′-ArH), 3.85 and 3.84 (s, 6H, $ArOCH_3$)
43	4-NO_2	4′-Cl	156–158	78	$C_{14}H_8ClN_3O_4$ (317.69)	52.59 52.93	2.72 2.54	13.42 13.23	1591 (C=N^+–O^-), 1516 (NO_2, asym), 1440 (=N^+(O^-)–O), 1350 (NO_2, sym)	8.35–8.30 (m, 2H, 3,5-ArH), 7.77–7.71 (m, 2H, 2,6-ArH), 7.50–7.42 (m, 4H, ArH)

Compd. No.	X	Y	M.p. (°C)	Yield (%)	Molecular formula (M_r) and mass (m/z)	Elemental analysis, found/calcd. (%)			IR (ν, cm^{-1})	^1H NMR (δ, ppm)
						C	H	N		
44	4-NO_2	4′-Br	170–172	30	$C_{14}H_8BrN_3O_4$ (362.14)	46.22 46.43	2.49 2.23	11.45 11.60	1585 (C=N^+–O^-), 1521 (NO_2, asym), 1444 (=N^+(O^-)–O), 1350 cm^{-1} (NO_2, sym)	8.34–8.29 (m, 2H, 3,5-ArH), 7.77–7.71 (m, 2H, 2,6-ArH), 7.65–7.60 (m, 2H, 2′,6′-ArH), 7.40–7.35 (m, 2H, 3′,5′-ArH)
45	4-NO_2	4′-F	127–128	26	$C_{14}H_8FN_3O_4$ (301.24)	55.66 55.82	2.42 2.68	14.22 13.95	1589 (C=N^+–O^-), 1519 (NO_2, asym), 1440 (=N^+(O^-)–O), 1350 cm^{-1} (NO_2, sym)	8.34–8.29 (m, 2H, 3,5-ArH), 7.77–7.72 (m, 2H, 2,6-ArH), 7.53–7.48 (m, 2H, 2′,6′-ArH), 7.21–7.13 (m, 2H, 3′,5′-ArH)
46	4-NO_2	4′-Me	146–148	80	$C_{15}H_{11}N_3O_4$ (297.27)	60.27 60.61	3.97 3.73	14.29 14.14	1586 (C=N^+–O^-), 1540 (NO_2, asym), 1439 (=N^+(O^-)–O), 1350 (NO_2, sym)	8.31–8.26 (m, 2H, 3,5-ArH), 7.78–7.72 (m, 2H, 2,6-ArH), 7.39–7.35 (m, 4H, ArH), 2.44 and 2.42 (s, 3H, $ArCH_3$)
47	4-NO_2	4′-SO_2Me	156–158	46	$C_{15}H_{11}N_3O_6S$ (361.34)	49.69 49.84	2.78 3.06	11.49 11.62	1600 (C=N^+–O^-), 1523 (NO_2, asym), 1444 (=N^+(O^-)–O), 1350 (NO_2, sym), 1311 (SO_2, asym), 1153 cm^{-1} (SO_2, sym)	8.37–8.31 (m, 2H, 3,5-ArH), 8.10–8.04 (m, 2H, 3′,5′-ArH), 7.76–7.71 (m, 4H, ArH), 3.13 and 3.11 (s, 3H, $ArSO_2CH_3$)

3.16 c. Organometallic complexes - Vanadium complex of Curcumin



Structure of bis[curcumino]oxovanadium (BCOV).

As advancement in the synthesis of organometallic complexes, we have chelated the vanadium centre with Curcumin. Curcumin, an active constituent of curcuma longa, has been reported to have very good anti-inflammatory, antipyretic and analgesic activity. Furthermore, this complex have been reported for its anti dieabetic activity. The concept of combining vanadium with a Curcumin ligand to form a compound that exhibits synergistic activity was appealing.

Synthesis of BCOV.

The chelated bis[curcumino]oxovanadium was prepared by reaction of curcumin (2 mole) and vanadyl sulfate (1 mole) in ethanol under alkaline conditions. The compound was isolated as a dark coloured complex. The vanadium content in the complex was determined by atomic absorption spectroscopy. It has one unpaired electron in the 3d orbital, characteristic of the vanadyl unit. It showed fairly strong V=O stretching vibration at 974 cm^{-1} in the IR spectrum.

Briefly, 400 mg of curcumin was dissolved in 100 ml of ethanol. A total of 100 mg of vanadyl sulfate pentahydrate was dissolved in 2 ml distilled water and the resulting solution was added drop wise to the refluxing solution of curcumin in ethanol. The reaction mixture was alkalized by addition of ammonia solution,

refluxed for 2 h in a water bath and concentrated. After cooling, the product crystallized out as dark coloured crystals (450 mg; melting point above 300 °C (d)). UV (MeOH): 416 nm and 234 nm; IR: 1591, 1512, 1280, 1160, 1125, 974 cm^{-1}. $C_{42}H_{38}O_{13}V$ requires C, 62.92; H, 4.78; V, 6.35. Found: C, 62.55; H, 5.12; V, 6.82.

3.16 d. Herbal drugs extracts: Following herbal drugs have been used many years from ancient time but their detailed pharmacology has not yet studied specially with reference to anti-inflammatory activity.

- Banaba-*Lagerstroemia speciosa L*— leaf extract(BNB)
- Ashwagandha- *Withania Sominifera*-root extract
- Lodhra- *Symplocos racemosus*-root extract
- Sariva- *Hemidesmus Indicus*-root extract
- Arjuna- *Terminalia arjuna*-Bark extract
- Pomegranate- *Punica Granatum*-Fruit extract(PME)
- Bitter Melon.- *Memordica Charantia*- fruit extract
- Tulsi- *Ocimum sanactum*- leaf extract
- Wheatgrass extract- *Elytrigia dasystachya*- whole plant extract

3.2 METHODS

3.21: ANTI-INFLAMMATORY ACTIVITIES

3.21.1 *In vitro* Assay

The *in vitro* potency of a drug is reflected by its IC_{50} value. This is the concentration at which the drug achieves 50% of its maximal inhibition of COX, and is usually expressed in molarity. The lower the IC_{50} value, the more potent the drug is. It has been proposed that the COX-2 selectivity of an NSAID can be defined by the ratio of the IC_{50} for COX-2 divided by the IC_{50} for COX-1 ($IC_{50\ COX-2}/IC_{50\ COX-1}$). NSAIDs with values < 1 would be said to have selectivity for COX-2; this means that a smaller dose of the drug is needed to inhibit COX-2 than was needed to inhibit COX-1. The smaller the number, the higher the selectivity for COX-2.

COX-1 and COX-2 Inhibition Assays

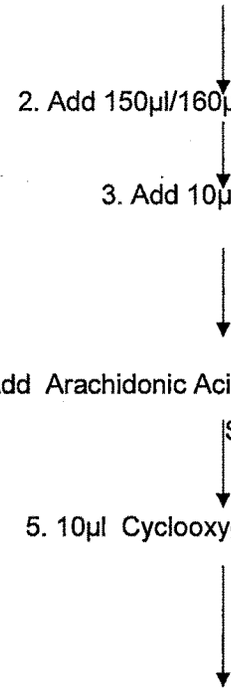
Principle of assay: The ability of compound to inhibit COX-1 and COX-2 enzyme activity was examined using the Cayman COX-1 and COX-2 colorimetric screening kit. This assay analyzes the peroxidase activity of the enzymes using *N*,*N*,*N'*,*N'*-tetramethylphenylenediamine(TMPD) as the reducing co-substrate.[173] The kit includes purified ovine COX-1 and COX-2 enzymes. Indomethacin was used as the positive control for COX-1 inhibition; Valdecoxib was used as the positive control for COX-2 inhibition. The assays were run according to the manufacturer's instructions.

Assay steps: All MCR synthetic compounds and semi synthetic compound BCOV were first tested for their COX-2 inhibition and COX-1 inhibition activity at 22μM and 88μM respectively. Herbal extracts were first tested at 100μg/ml for both COX-1 and COX-2. The compounds showed good inhibition at these concentrations were tested at lower concentrations to find IC_{50}.

Sample Preparation- all synthetic compounds were dissolved in DMSO e.g. 25mg compound dissolved in 0.25ml and serially diluted in PBS(pH-7.4). For herbal extract 500mg of drug dissolved in methanol and further diluted in PBS(pH-7.4).

Assay steps

1. Add 10µl test substance/Std./Vehicle in 96 well plate.



2. Add 150µl/160µl of Assay buffer

3. Add 10µl of Heme

Shake the plate few seconds and incubate 5mins at 25° C.

4. Add Arachidonic Acid (20 µl) + TMPD 20µl

Shake the plate few seconds and incubate 5mins at 25° C.

5. 10µl Cyclooxygenase Enzyme

Shake the plate few seconds and incubate 5mins at 25° C.

6. Read the absorbance**(Plate reader, Molecular devices, USA)** at 590nm

Type	Test sol.	Assay Buffer	Heme Sol.	AA+ TMPD	Cox-1/2	Total Vol.
Blank	-	160µl	10µl	40µl	10µl	220µl
Test sub	10µl	150µl	10µl	40µl	10µl	220µl
STD	10µl	150µl	10µl	40µl	10µl	220µl

Selection of compounds for further study:

Only 32 synthetic compounds out of 93 showed some good COX-2 over COX-1 inhibition. Semi synthetic compound BCOV showed better COX-2 inhibition compared to its base Curcumin. Out of 9 herbal drugs *BNB and **PME relatively more selective COX-2 inhibition. Those compounds which showed promising *in vitro* results were further studied for acute *in vivo* anti-inflammatory model of carrageenan induced rat paw edema. They are as follow:

Synthetic compounds:
MCR-95, MCR-101, MCR-163, MCR-175, MCR-207, MCR-189, MCR-192, MCR-193,MCR-190, MCR-179, MCR-180, MCR-240, MCR-241, MCR-243, MCR-242, MCR-262,MCR-263, MCR-264, MCR-265, MCR-273, MCR-292, MCR-293, MCR-295, MCR-296, MCR-316, MCR-320, MCR-322, MCR-323, MCR-327, MCR-333, MCR-349, MCR-364, MCR-363.

Semi synthetic compound: BCOV

Herbal Drugs Extracts:
BNB, PME
***BNB- Banaba-*Lagerstroemia speciosa L*— leaf extract**
****PME- Pomegranate- *Punica Granatum*-Fruit extract**

3.21.2 *In vivo* assay for efficacy

Anti-inflammatory studies were carried out in two different categories.

a. Acute Inflammation Model -Carrageenan Induced Paw Edema

b. Chronic Inflammation Model- Adjuvant induced arthritis, TNBS induced colitis in rats

Acute model of inflammation - carrageenan induced rat paw edema

The Carrageenan induced rat paw edema assay was carried out using procedures described by *Otterness and Moore.*[175,176] Paw edema was induced by injecting 0.05 ml of a 1% carrageenan saline solution into the hind paw of male Sprague-Dawley rats (180– 280 g), five animals per dose group. Prior to experiments, the rats were allowed free access to food and water. 32 MCR compounds/ BCOV / PME/BNB / vehicle (1% CMC) were administered orally 1 h before the carrageenan injection and paw volume was measured by water displacement using **Plethysmometer(Ugo Basile)** at intervals 0hr and 3hr of carrageenan injection. The formula for computing percent inhibition of edema is: difference between the average increase in the paw volume of control group and treated group divided by average increase in the paw volume of control group times 100.

32 Selected compounds including semi synthetic BCOV from *in vitro* studies were tested for model of acute inflammation-carrageenan induced paw edema at doses 25mg/kg/p.o.(MCR compounds/BCOV)/ 500mg/kg/p.o.(herbal drugs). The compounds listed below showed good inhibition of edema, they were further tested at lower doses to find out ED_{50}.

MCR 363, BCOV, MCR-95,BNB, PME

Selection of compounds:

From *in vitro* COX inhibition and *in vivo* acute carrageenan induced paw edema, finally four compounds MCR 363, BCOV, BNB, PME were selected for the further screening for chronic inflammation models and toxicological studies including safety pharmacology.

MCR 364 and MCR 207 showed good COX-1 /COX-2 profile but not selected for further studies because they didn't show any inhibition in paw edema model. MCR-95 was also rejected for the further screening, it showed good inhibition of carrageenan foot pad edema but showed very poor COX-1/COX-2 profile.

3.12.1b Chronic models of inflammation

Adjuvant induced arthritis[176]

Sprague Dawley rats (150-200 g), were weighed, marked and assigned to different groups as per given below. Following listed compounds were tested for anti-inflammatory activity in chronic model of inflammation- adjuvant-induced arthritis.

Sr. No	Test Substance	Dose mg/kg/p.o./day	Adjuvant Inj. at 0 day	Duration
1	Normal Control	Vehicle	--	21 days
2	Adjuvant Control	Vehicle	+	21 days
3	Indomethacin	5	+	21 days
4	MCR 363	25	+	21 days
5	BCOV	25	+	21 days
6	BNB	500	+	21 days
7	PME	500	+	21 days

Adjuvant protocol: Adjuvant was made by grinding ***Mycobacterium butyricum*** in a mortar and adding to it light mineral oil so that the final concentration was 5mg/ml. Rats received a sub plantar injection of 0.1 ml of adjuvant in the left hind paw by inserting a 26-gauge 0.5-inch needle between the second and third digit into the dorsum of the hind paw.

Foot volume: Foot volumes were determined by Plethysmometer(Ugo Basile) before the administration of adjuvant. Change in foot volume was calculated as the difference between day 0 and the day of sacrifice (Day 21). Body weights and paw volumes of both hind feet were taken before injection of adjuvant (day 0). Each rat was then injected with adjuvant in the left hind foot. A group of non injected rats served as normal age-matched controls. As showed above in the table the animals were orally administered either vehicle (1% CMC), or MCR363 (25mg/kg/p.o.) or BCOV (25mg/kg/p.o.) or BNB (Banaba extract) (500mg/kg/p.o.) or PME(Pomegranate extract) (500mg/kg/p.o.) and Sulphasalazine (20mg/kg/p.o.) groups from 0-21 days.. On various days after adjuvant injection, body weights and foot volumes were recorded. On 21st day the spleens, thymus and adrenal were removed weighed after sacrificing the rats. Both hind feet were removed proximal to the tibio-tarsal joint, and lateral to medial radiographs were made. % inhibition in paw volume calculated from following equation: difference between the average increase in the paw volume of control group and treated group divided by average increase in the paw volume of control group times 100.

b. TNBS (trinitrobenzesulfonic acid) induced inflammatory colitis in rats.[177]

Male Sprague Dawley rats (140-160 g), were weighed, marked and assigned in 7 groups of 5 animals each. Grouping and treatment given as per table below.

Sr.No	Test Substance	Dose mg/kg/p.o./day	Treatment Duration
1	Normal Control	vehicle	14 days
2	TNBS Control	vehicle	14 days
3	MCR 363	25	14 days
4	BCOV	25	14 days
5	BNB	500	14 days
6	PME	500	14 days
7	Sulphasalazine	20	14 days

0.85 ml enema containing 30 mg TNBS dissolved in 50% ethanol was prepared. Rats were anaesthetized with ether. A silicon plastic tube with a 2 mm diameter was inserted into the rat colon 8 cm distant from the anus, 0.85 ml enema containing TNBS 50% ethanol was given using 1ml syringe. Normal control animal received 0.85 ml of 50% ethanol. From day 0-14 as showed above in the table the animal were dosed orally either vehicle (1% CMC), MCR363(25mg/kg/p.o.),BCOV(25mg/kg/p.o.), BNB 500mg/kg/p.o., PME 500mg/kg/p.o., Sulphasalazine(20mg/kg/p.o.) to respected group of animals.

Evaluation of the degree of injury and inflammation:

Bodyweight: Rats were weighed daily were examined each day. Gross morphology and histological lesions: The intestine, from the anus to the ileocecal colon, was dissected and incised along the mesentery. Five samples (2 × 10 mm) were obtained from the colon 1, 3 and 8 cm distant from anus and transverse and ascending colon, respectively. In areas of severe inflammation or ulceration, additional biopsies (at least one site) were taken. Samples underwent routine wax embedding, slicing (4 μm) and hematoxylin and eosin staining to observe histological changes and to score these(0-4) changes according to the criteria given in, which were modified from the criteria proposed by Vickers *et al.*[203]

Neutrophil myeloperoxidase (MPO) activity assay: Each 200 mg sample of distant colon tissue was treated with 1 mL of 0.2% 16-alkyl-3-methyl-amine bromide, homogenized, frozen and thawed three times, then centrifuged (10 min, 2000 r.p.m.) to obtain 0.1 mL supernatant. The mean shift in optical density (OD) within 5 min at 655 nm was assessed using an ultraviolet spectrophotometer, with tetramethyl diphenylamine as the substrate. One enzyme activity unit was defined as a shift of 1.0 OD/min.

3.31 Analgesic activity: Two different models tail immersion method in mice and acetic acid induced writhing method used to determine analgesic activity of MCR363, BCOV,BNB, PME.

a. Tail immersion method in mice

In this method, analgesia was assessed according to the method of Di Stasi et al. (1988)[178] i.e. tail immersion method. Male mice with a weight between 20 and 25 g were used. The animals were randomly divided in to five group(n=5) and dosed p.o. route as per given below.

Test groups: Control group(Vehicle -1% CMC),
MCR 363(25mg/kg/p.o.),
BCOV(25mg/kg/p.o.),
BNB(500mg/kg/p.o.),
PME(500mg/kg/p.o.)

Drug Preparation: Test substance listed above were suspended in 1% CMC and administered to the groups of mice in a single oral dose by gavage needle before one hour of tail emersion. The control group received 1% CMC.

The animals were placed into individual restrainer leaving the tail hanging out freely. The lower 3cm portion of the tail was marked. This part of the tail was immersed in a beaker of freshly filled water of exactly 55 °C. Within a few seconds the mice reacts by withdrawing the tail. The reaction time was recorded in seconds by a stopwatch. After each determination the tail was carefully dried. The reaction time was determined before and periodically after oral administration of the test substance, e.g. at 0h and 3h. The cut off time of the immersion was 15s. The withdrawal time of untreated animals was between 1 and 5.5s. A withdrawal time of more than 6s was regarded as a positive response.

The criterion for analgesia was a post-drug latency, which was greater than two times to average pre-drug latency. Tail flick latency difference (TFLD) or mean increase in latency after drug administration was used to measure the analgesia produced by test and standard drugs. Analgesia TFLD was calculated as- Post-drug tail flick latency - Pre-drug tail flick latency.

b. Peripheral analgesic activity-writhing tests[179]

Pain is induced by injection of irritants into the peritoneal cavity of mice. The animals react with a characteristic stretching behavior which is called writhing. An irritating agent such acetic acid was injected intraperitoneal to mice and the stretching reaction was evaluated.

Test Groups: Acetic Acid Control(1% CMC)
MCR-363(25mg/kg/p.o.),
BCOV(25mg/kg/p.o.),
BNB(500mg/kg/p.o.),
PME(500mg/kg/p.o.)
Diclofenac(20mg/kg/p.o.)

Male mice weighed between 20 and 25 g were divided in to six groups containing five animals in each and dosed p.o. as per above listed compounds. Test drugs listed above were administered one hour prior to 0.1ml 1% acetic acid i.p. administration. Immediately after the acetic acid injection the mice were placed individually into glass beakers and five min allowed to elapse. Then observed for a period of ten min for the number of writhes was recorded. For scoring purposes, a writhe was indicated by stretching of the abdomen with simultaneous stretching of at least one hind limb. The formula for computing percent inhibition was: average writhes in the control group minus writhes in the drug group divided by writhes in the control group times 100%.

3.31 Antipyretic effect: Endotoxin induced pyresis in rats[180]

Male Sprague dawley rats(150-200g) divided in to six groups of five animals each were fasted for 16 to18 hrs before use. At zero hr the animals were placed temporarily in restrainer and the resting rectal temperature was recorded using a flexible temperature probe connected to Bio-Pac data acquisition system. The same probe and system used for all animals to reduce experimental error. The animals were returned to their respected cages after the temperature measurement. At zero hr, the rats were injected i.p with either saline or Lipopolysaccharide(LPS)(0.36 mg/kg). At five hr post LPS injection, first group

was treated with vehicle (1% CMC) was served as control group, while other groups were received treatments given below. At 7 hr post LPS injection the rectal temperature was measured to determine the rise in temperature was reversed in treatment group as compared to LPS control group. Percent reversal (antipyretic activity) was calculated using the rectal temperature obtained at 7hrs taking this value in the vehicle control group as zero reversal.

Treatment groups: LPS Control(Vehicle- 1% CMC),
MCR-363(25mg/kg/p.o.),
BCOV(25mg/kg/p.o.),
BNB(500mg/kg/p.o.),
PME(500mg/kg/p.o.),
Paracetamol(25mg/kg/p.o.)

3.22 SAFETY PHARMACOLOGICAL STUDIES:

3.22.1 Gastrointestinal Tract

In vivo assay for Ulcerogenicity[180, 181]

Gastric irritation properties of orally administered compounds were evaluated in fasted rats.

Fasted male Sprague-dawley rats (150-200g) were randomly divided in to five group (n=5). Following listed compound were screened for it's ulcerogenic effect.

Treatment groups: Normal Control (1% CMC)
MCR-363(100mg/kg/p.o.),
BCOV(100mg/kg/p.o.),
BNB(1000/kg/p.o.),
PME(1000/kg/p.o.)

Animals were dosed orally above mentioned test compounds or vehicle(1% CMC, 1 ml/100g body wt.) Four hours later, the animals were euthanized and the stomach excised along its greater curvature. After rinsing with normal saline, observed for ulcers. The number of ulcers was counted using a magnifying glass and the diameter of the ulcers was measured by following the scoring method of Suzuki et al[181].

Score 1: pinpoint lesion.

Score 5: Maximal diameter of 1–3 mm.

Score 10: An ulcer over 5mm in diameter.

Score 25: A perforated ulcer.

3.22.2 Cardiovascular system

a. CVS safety studies[182] (*In vivo*):

Hartley guinea pig with weight 300-400 g was used for this study. The g. pig was anaesthetized with 1.5 g/kg urethane i.p. A tracheotomy was performed and the guinea pigs were incubated on ventilator, breathing spontaneously throughout the experiment. Rectal temperature was monitored and maintained between 37.8 and 38.2 °C using infrared lamps. A polyethylene catheter was inserted into the carotid artery and attached to a pressure transducer. A Millar Micro-tip catheter pressure transducer was inserted into the contra lateral carotid artery and advanced to the left ventricle, allowing contractile parameters to be recorded. ECG was recorded by inserting needle electrodes according to standard limb leads after Einthoven I–III subcutaneously.

Animal Species:	Guinea Pig
Strain:	Harley
Gender:	Male
Rout of Administration:	p.o.
Test substance:	MCR-363(100mg/kg/p.o.), BCOV(100mg/kg/p.o.), BNB(1000mg/kg/p.o.), PME(1000g/kg/p.o.)
Duration of Study:	5-8 hrs

Drug Preparation and administration: Test substance suspended in 1% CMC was administered to the guinea pig in a single oral dose by gavage needle half hour before the monitor of blood pressure and ECG.

Protocol: Pressure transducers (Bio-Pac, U S A) were calibrated and a 30-min equilibration period for stabilization of haemodynamic parameters was kept. A vehicle study (1 % CMC) was performed to obtain control values of the cardiovascular parameters under placebo conditions.

Data acquisition: Using the acquisition software Bio Pac data acquisition system, USA, the haemodynamic (arterial blood pressure, Heart rate) and ECG parameters were recorded continuously.

b. Isolation, mounting and stabilization of isolated aortic rings[183]

The thoracic aorta of rats was isolated immediately and carefully cleaned of fat and connective tissues. The aorta was cut in to rings of 3mm width, extreme care was taken not to stretch or damage the luminal surface of the aorta to ensure the integrity of endothelium. Aortic rings were suspended between two 'S' shaped platinum loops in jacketed organ bath containing 20ml krebs bicarbonate solution (pH 7.4) maintained at 37±0.5 °C temperature and continuously aerated with 95% oxygen and 5% carbon dioxide. The composition of Krebs solution (mM) was NaCl 118, KCl 4.7, $CaCl_2$ $MgSO_4$ 1.2, $NaHCO_3$ 22.0 and Glucose 11.0. The rings were connected to isometric force displacement transducer connected to Gemini pen recorder(UGO-BASILE, Italy). The rings were maintained under 2 g and equilibrated for 90 min before initiating experimental protocol. During this period, the krebs solution was changed at every 15 min interval. After the equilibration period rigs were maximally contracted with phenylephrine(PE, 1µM) to test their contractile capacity, three recordings were carried out to find out constant and reproducible contraction. The presence of functional endothelium was assessed by the ability of acetylcholine(Ach, 0.1 µM) to induced more than 60% of relaxation of rings precontracted sub-maximally with PE. Aortic rings were considered denuded when there was less than 10 % relaxation to Ach. At the third and fifth hour, a new CRC was obtained with Phenylephrine plus Vehicle 10% DMSO, test drugs MCR 363(1µ-1mM),BCOV(1µ-1mM), BNB and PME at concentrations of 1-20 mg/dl, the test compounds were dissolved in 10% DMSO in water.

3.22.2 Central nervous system safety studies:

Effects of the test substance on the central nervous system can be assessed by several models e.g. motor activity, behavioral changes, coordination, sensory/motor reflex responses and body temperature. Following test drugs and doses have been used for all CNS safety studies.

Animal Species:	Mice
Strain:	CD1
Gender :	Male
Treatment groups:	MCR-363(100mg/kg/p.o.),
	BCOV(100mg/kg/p.o.),
	BNB(1000/kg/p.o.),
	PME(1000/kg/p.o.)

Drug Preparation and Administration : compounds suspended in 1% CMC was administered to the groups of mice in a single oral dose by using gavage feeding needle.

a. Locomotor activity in mice[186]

Male mice were divided into five groups of five mice each. Mice were administered either MCR363 (100mg/kg/p.o.), or BCOV(100mg/kg/p.o.),or BNB(1000 mg/kg/p.o.), or PME(1000 mg/kg/p.o.) or Diazepam(2mg/kg/i.p.) Animals in the remaining group received 1%CMC (10 ml/kg) as control. 1hr later the animals were transferred individually to actophotometer and motor activity was recorded for 10mins.

b. Observational assessment [187]

A systematic, quantitative procedure assessing the behavioral state of mice for the evaluation of drugs has been described by Irwin. The method is applied in the beginning of pharmacological screening to detect psychotropic activities. It allows identifying and differentiating the profile pattern of various classes of pharmacological agents. Furthermore, observational assessment allows into the safety and potential toxicity profile of a new drug.

Five animals were used for each treatment group. One group of 5 animals received the vehicle only, served as control group. 1hr after drug administration,

the animals were closely observed for following parameters and compared to the vehicle control group:

Effects on CNS

- spontaneous motor activity
- restlessness
- grooming behavior
- squatting
- staggering
- ataxic gait
- lying flat on the belly
- lying flat on the side
- lying flat on the back
- sleeping
- narcosis
- bizarre behavior
- timidity
- Straub phenomenon
- writhing
- tremors
- twitches
- opisthotonus
- clonic convulsions
- tonic convulsions
- rolling and jumping

Effects after manipulations

- auditory stimulus response
- escape after touch
- righting reflex
- paresis of hind limbs
- paresis of forepaws

- catalepsy in induced positions

Effects on reflexes

- pinna reflex
- corneal reflex
- pain following stimulation

Effects on autonomic nervous system

- pupil diameter(constriction or dilatation)
- eyelids
- (closure or exophthalmus)
- secretion of sweat
- salivation
- lacrimation
- cyanosis
- piloerection
- defecation
- urination

The number of deaths was counted during the first 24 h and after 7 days in order to evaluate acute and late toxicity. Arbitrary scores were chosen for each symptom.

c. Pentobarbital sleeping time in mice[186]

The test was performed on five groups of five mice each. Treatment groups received vehicle(1%CMC/p.o.), MCR-363(100mg/kg/p.o.), BCOV(100mg/kg/p.o.), BNB(1000/kg/p.o.), PME(1000/kg/p.o.) and (1 mg/kg/i.p.) prior to 1hr of pentobarbital sodium injection(40 mg/kg/i.p.). Each mouse was observed for the onset and duration of sleep, with the criterion for sleep being loss of righting reflex. The interval between loss and recovery of righting reflex was used as the index of hypnotic effect.

d. Test for motor co-ordination (rota-rod test) in mice[184]

Rota-rod treadmill device (Ugo Basile no. 7600, Varese, Italy) was used for this purpose. The mice were placed on a horizontal rotating rod set at a rate of 16 revolutions per min. Mice that were able to remain on the rod longer than 180 s were selected and divided into five groups of five mice per group. Four groups of the animals received test substances either MCR 363(100mg/kg/p.o.), BCOV(100mg/kg/p.o.), BNB(1000/kg/p.o.), PME(1000/kg/p.o.), while the remaining group received vehicle 1% CMC to serve as control. 1hr later, each mouse was placed on the rod and the time spent by mice on rota rod was recorded.

3.3 TOXICITY STUDIES

3.3.1 Acute toxicity studies- OECD guideline-420 [188, 189]

Acute toxicity describes the adverse effects of a substance which result either from a single exposure or from multiple exposures in a short space of time (usually less than 24 hours). To be described as acute toxicity, the adverse effects should occur within 14 days of the administration of the substance.

Acute toxicity is distinguished from chronic toxicity, which describes the adverse health effects from repeated exposures, often at lower levels, to a substance over a longer time period (months or years).

Animal Species:	Mice
Strain:	CD1
Gender :	12 males / dose
Rout of Administration:	p.o.
Dose:	0.3-5 g/kg
Volume dose:	10ml
Test substance:	Control, MCR 363, BCOV, BNB, PME
Duration of Study:	1 day treatment and 14 days observation

Solution Preparation: Test substance suspended in 1% CMC was administered to the groups of mice in a single oral dose by gavages using a feeding needle.

This study is in accordance with the OECD guideline-420 for the testing of chemicals. The control group received an equal volume of the vehicle. Twelve mice were used for each dosage ranging from 0.3-5 g/kg. They were deprived of food, but not water 16–18 h prior to the administration of the test suspension. Observations of toxic symptoms were made and recorded systematically at one, two, four and six hours after administration. Finally, the number of survivors was noted after 24h and these animals were then maintained for a further 13 days with observations made daily. At the conclusion of the experiment, all surviving animals were sacrificed and their organs such as liver, lungs, heart, spleen, adrenals, kidneys, testes and ovaries were excised for the pathological observations of these tissues were performed on gross bases.

3.33.2 Sub acute toxicity (pilot study)[190]- Due to the technical limitations only some part of sub acute toxicity study has been performed.

This study is in accordance with OECD guideline-407[190] for the repeated dose 28-day oral toxicity study in rodents.

Animal Species:	Rat
Strain:	Sprague dawley
Gender :	5 males + 5 females
Rout of Administration:	p.o.
Treatment Groups:	Vehicle(1% CMC)
	MCR-363(100mg/kg/p.o./perday),
	BCOV(100mg/kg/p.o./per day),
	BNB(1000 mg/kg/p.o./per day),
	PME(1000 mg/kg/p.o./per day)
Duration of Study:	28 Days

Solution Preparation : Test substance suspended in 1% CMC was administered to the groups of mice in a single oral dose by gavages using a feeding tube.

Experimental Design and Conduct:

Ten healthy rats, 5 males and 5 females in each treatment group, were acclimatized to laboratory conditions for 7 days prior to initiation of dosing. They were randomly assigned to cages and the individual animal was fur marked with picric acid. The females were nulliparous and nonpregnant. The rats were deprived of feed for 16 hours before and 3 hours after administration of the test sub-stance. The test substance, suspended in 1% CMC, 10ml/kg was administered by gavage to rats using a ball-tipped intubation needle fitted onto a syringe. The doses were selected on the bases of maximal therapeutic doses from the *in vivo* studies.

Observations of pharmacotoxic signs were made at 10, 30, 60, and 120 minutes and at 4 and 6 hours after dosing during the first day and daily thereafter for 28 days. The time of onset, intensity, and duration of these symptoms, if any, was recorded. All animals were observed twice daily for mortality during the 28-day period of study. The weight of each rat was recorded on day 0 and at weekly intervals throughout the course of the study and means body weights were calculated. The quantity of food consumed by groups consisting of rats was recorded weekly and the food consumption per rat was calculated for control and treatment groups. The eyes of control and animals from different groups were examined prior to the initiation of the dosing in week 4 of the study period on day 28.

At the end of the 28-day period the animals were fasted overnight. The following morning, each animal was heparinized and blood samples were collected from the orbital sinus. The hematological parameters hemoglobin concentration (Hb), mean corpuscular volume (MCV), total erythrocyte count (RBC), reticulocyte concentration (Rt), mean corpuscular hemoglobin (MCH), hematocrit (HCT), and total and differential leucocytes count were determined. The rats were sacrificed on day 29 by spinal cord dislocation. Macroscopic

observation of all animals was carried out and the weights of lung, liver, kidneys, adrenals, heart, brain, ovary and testes were recorded. Since kidney and liver are the target organ for the toxicities of NSAID, histopathological observations were carried out only in kidney and liver of control and different treatment group to find out any damage microscopically. Tissue samples were preserved in 10% neutral buffered formalin.

3.4 Statistical Analysis

All the data are expressed as mean ±SEM. Statistical significance between more than two groups was tested using one-way ANOVA followed by the Bonferroni multiple comparisons test or unpaired two-tailed Student's *t*-test as appropriate using a computer- based fitting program (Prism, Graph Pad). Differences were considered to be statistically significant when $p < 0.05$.