5. DISCUSSION

The present investigation deals with assessment of the compounds for their *in vitro* and *in vivo* activities with respect to their anti-inflammatory, toxicological studies including safety pharmacological studies. Valdecoxib and Indomethacin were taken as reference standards in all *in vitro* and *in vivo* studies.

Initial assessment of the potency and selectivity of test compounds with respect to COX-1 and COX-2 were determined from IC_{50} values, expressed as the concentration of compound required to inhibit 50% of the initial rate of (i) oxygen uptake or (ii) TMPD oxidation. The compounds were tested for their ability to inhibit COX-1 and COX-2 by the colorimetric COX (ovine) inhibitor screening assay. This *in vitro* screening, compounds were assayed against COX-2 at 22μM and COX-1 at 88μM Herbal drugs were tested at 100μg/ml. For the potent selective COX-2 inhibitors, full IC_{50} were then determined. Compounds that showed promising COX-2 inhibitory activity was further screened for acute *in vivo* anti-inflammatory activity using the carrageenan induced rat paw edema method.

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used for the treatment of pain, fever, and inflammation. Conventional NSAIDs act as nonselective inhibitors of cyclooxygenase (COX) enzymes, which catalyze the formation of prostaglandins (PGs) from Arachidonic acid. There are at least two mammalian COX isoforms.[13,14] COX-2 is induced in response to proinflammatory conditions, while COX-1 is constitutive and responsible for the maintenance of physiological homeostasis, such as gastrointestinal integrity and renal function. Traditional NSAIDs such as aspirin, Indomethacin, and diclofenac are nonselective inhibitors and are associated with gastric ulceration, bleeding, and renal function suppression.[26] Generally, prostacyclin and thromboxane normally balance each other's opposing effects. Introducing the selective COX-2 inhibitor may bias the balance dangerously toward thromboxane, raising blood pressure, possibly hardening arteries and certainly promoting heart-attack and stroke-causing clots in some patients.[17,] This could explain the cardiotoxicity caused by Rofecoxib (Vioxx),[109] and subsequently withdrawal of the drug from market.

In the present *in vitro* study, we have studied the COX-isozyme selectivity of the novel COX-2 inhibitors MCR series compounds, an organometallic compound -BCOV and some herbal drugs by comparing their inhibitory effects with standard drugs Indomethacin, Valdecoxib by Cayman colorimetric COX inhibition screening assay.[173,174] Selective COX-2 inhibitors have been designed with the aim of developing a new class of analgesic and anti-inflammatory drugs with fewer side-effects than conventional NSAIDs, particularly COX-1-dependent GI complications. The first selective COX-2 inhibitors approved by the FDA and the EMEA for the treatment of RA and OA,[146] Celecoxib and Rofecoxib, are diarylheterocyclic derivatives. Structure-activity studies indicated that a cis-stilbene moiety containing a 4-methylsulfonyl or sulfonamide substituent in one of the pendant phenyl rings was required for COX-2 selectivity.[174] In the present study, efforts are made to keep same pharmacophore -4-Di(4-methoxyphenyl)-1,2,5-oxadiazole *N*-oxide which is important for cox-2 inhibition but introduction of different functional groups at para and ortho positions e.g. -H, -Cl, -Br, -F, -CH_3, -OCH_3 , -NH_2, -NHAc, -$NHSO_2Me$, -NO_2, -SOMe, -SH, -SO_2NH_2 to reduce GI and CVS side effects.

A group of MCR series compounds were designed such that the COX-2 SO2Me pharmacophore was located at the para-position of either the C-3 or the C-4 phenyl substituent on the central pyran-2-one ring. In addition, the substituent at the para-position of the C-6 phenyl ring was varied (H, Me, Et, CF3, OMe, OEt, F, SMe) to determine the effect of steric and electronic substituent properties on COX-2 inhibitory potency and selectivity. Structure-activity relationship (SAR) data for the title compounds (IC_{50} values) were acquired by evaluating their *in vitro* ability to inhibit the COX-1 and COX-2 isozymes.

The current study investigated the effect of novel synthetic compounds and herbal drugs on inhibition of cyclooxygenase activity and COX-2 expression. Amongst 103 compounds MCR-207, MCR-363, BCOV, BNB extract, PME extract have shown some promising inhibitory activity on COX-2 and COX-1, favoring COX-2 inhibition by more than four-fold. However, because this difference is only four-fold, it would not likely have the gastric sparing properties of highly selective COX-2 inhibitors, such as Rofecoxib, but it may also not

possess the cardiovascular risks that have been reported for this drug.[28] We have found that Valdecoxib and some MCR series compounds are at least two orders of magnitude more potent in inhibiting monocyte COX-2 than platelet COX-1. This finding is consistent with substantial sparing of platelet and gastric mucosa COX-1 activity, as assessed *in vitro* and *in vivo*, respectively, at therapeutic oral dosing.[191,192,193]

The development of novel COX-2 inhibitors with improved biochemical selectivity over that of commercially available coxibs may have two distinct potential advantages. In principle, it should lead to a clear-cut separation of COX-2- from COX-1-dependent effects, by virtue of maximizing the likelihood of exposed patients being in the 80-100% range of COX-2 inhibition and in the 0-20% range of COX-1 inhibition throughout the dosing interval. Whether this will allow using higher coxib doses for improved efficacy remains to be established, given the dose-dependence of mechanism-based renal effects associated with these agents.[194] Secondly, it could diminish the probability of the COX-2 inhibitor interfering with permanent inactivation of platelet COX-1 by low-dose aspirin, in patients requiring anti-inflammatory and antiplatelet therapy.[15] Recent studies suggest that the likelihood of selective COX-2 inhibitors sharing this pharmacodynamic interaction of conventional NSAIDs is inversely related to their COX-2 selectivity.[16] However, the controversial cardiovascular findings of the VIGOR trial have led to discussion of whether the vascular consequences of endothelial COX-2 inhibition are modified by unopposed platelet TXA_2 production.[21] At least three possible explanations can be entertained in accounting for the statistically significant difference in MI rates between rofecoxib and naproxen (0.4 vs 0.1%, respectively), as reported by VIGOR trial.[195] First, a cardio protective effect of naproxen: but, there is no convincing evidence that conventional NSAIDs reduce the risk of MI.[195] Second, a thrombogenic effect of coxibs: but, the size of the effect is not biologically plausible if it is due to incomplete inhibition of a single mediator of 'thromboresistance', i.e. PGI_2.[69] Moreover, such an explanation is not substantiated by CLASS results,[69] though a smaller coxib effect cannot be excluded. Third, the play of chance: the apparent difference in VIGOR might represent uneven distribution of a small number of

events occurring over a short time frame in a low-risk population; a metanalysis of all coxib trials might address this possibility.

However, it should be mentioned that selective COX-2 inhibition may have potential beneficial effects in addition to low-dose aspirin in the setting of acute coronary syndromes. Thus, COX-2 expressed in inflammatory cells may account for aspirin-resistant TXA_2 biosynthesis in acute coronary syndromes. Baseline urinary excretion of 11-dehydro-TXB_2 (a major enzymatic metabolite of TXA_2) predicted the future risk of myocardial infarction (MI) or cardiovascular death in a subgroup of high-risk aspirin-treated patients participating in the HOPE trial.[196]

Using the colorimetric *in vitro assay*, we have shown that the new compounds, MCR- 363 has higher COX-1/COX-2 IC_{50} ratios than Valdecoxib (Table 12).The manipulation of the ring system of rofecoxib and celecoxib that is fused to the stilbene framework has led to the synthesis of valdecoxib, a diarylisoxazole derivative which was recently approved by the FDA. In the present study, MCR-363 resulted in 65 fold potency towards monocyte COX-2 than platelet COX-1 that is higher than Valdecoxib and celecoxib. BCOV has shown good cyclooxygenase inhibition enzyme inhibition to both enzyme COX-1 and COX-2, its COX-2 selectivity ratio is 3.33 shows higher than its parent compound Curcumin which has COX-2 selective ratio 1.08. Amongst the herbal groups BNB and PME extract has shown impressive *in vitro* results their COX-1/COX-2 selectivity ratio were 2.01 and 1.48 respectively. That was the reason they have taken for further *in vivo* and secondary pharmacological screening.

Conventional anti-inflammatory drugs are tested in a range of animal models designed to demonstrate a direct overall anti-inflammatory effect. Many rely on the injection of an irritant or inflammatory substance, such as croton oil, carrageenan, poly-L-arginine or lipopolysaccharide. The carrageenan rat paw oedema model is well established and widely used to demonstrate the effects of anti-inflammatory agents. It remains a standard model for new agents; Brand *et al.*[197] used it in their evaluation of capsaicin analogues, while Kumakura *et al.* [198] used it to demonstrate the effects of a prodrug of indomethacin.

In the present study reported here, amongst the MCR series compounds many compounds were able to reduce the inflammation provoked by Carrageenan in the rat paw model. MCR-363, MCR-364 reduced the inflammation more than any

other oxadiazole series compounds., at 25mg/kg/p.o. dose almost completely suppressing the edema produced by carrageenan. The semi synthetic drug BCOV has slightly better activity than the base Curcumin. From the herbal drugs, only BNB and PME have able to show some better inhibition of inflammation, while others failed to inhibit the edema. The calculated ED_{50} values for synthetic compounds MCR 363, BCOV are 13.9±2, 41.1±5 mg/kg/p.o. respectively. The ED_{50} for BNB and PME came 569±31 and 632±27 mg/kg/p.o. respectively. The ED_{50} value for standard drug Valdecoxib is 10.2±3mg/kg/p.o..

LPS-induced pyresis NSAID, such as diclofenac, are antipyretic in both human and animal models of pyresis.[199] The oral treatment of MCR 363, BCOV and PME have shown atlease more than 50% of temperature reversal at doses 25, 25 and 500mg/kg/p.o. respectively which is comparable to paracetamol(dose 10mg/kg/p.o.). It should be noted that both in the rat paw edema assay and in MCR 363, BCOV and PME the endotoxin-induced pyresis assay, was able to reverse completely both the nociceptive and the pyretic responses. This would suggest that, at least in these models, COX-2-derived PGs are largely responsible for both the acute nociception and the endotoxin-induced pyresis.
Experimental investigations revealed that the MCR 363 and BCOV have dose-dependent activity against inflammation as revealed in the carrageenan models. According to Vinegar et al[200], Antonio and Brito[201] in the carrageenan model, the early phase (1–2 h) is mainly mediated by histamine, serotonin and the increase of PG synthesis in the surroundings of the damaged tissues while the late phase is mainly mediated by bradykinin, leukotrienes, polymorphonuclear cells and PGs produced in tissue macrophages. In this experiment, the suppression of inflammation at the early phase of inflammation can be contributed by PG synthesis inhibition was shown in *in vitro* cox inhibition.

Further, tail flick test and acetic acid induced writhing test revealed that MCR 363 and BCOV have slight analgesic activity via peripheral pathway showed 35% and 30 % inhibition in acetic acid induced writhing, but they failed to show any activity on tail flick model, indicates they don't have any analgesic activity via CNS pathway. MCR 363 can also suppress acute pain. However, the anti-inflammatory activity is comparative with valdecoxib. The analgesic activity shown only in the tail flick test reveals that the activity is supraspinally

mediated[202] and can be brought about by the PG synthesis inhibition activity of MCR 363 and BCOV.

In the acetic acid induced writhing test, the pain in the early phase is caused due to the direct stimulation of the sensory nerve fibers by acetic acid while the pain in the late phase is due to inflammatory mediators, like histamine, PGs, serotonin and bradykinin.[202] The pain suppression at the early phase by test drugs as well as Diclofenac may be due to a local effect caused by the membrane stabilizing activity, because membrane stabilizing agents produce local anesthesia reducing the sensation of acute pain. The considerably higher pain suppression at the late phase than the early phase could be due to the concerted action of PG synthesis inhibition, membrane stabilization and antihistamine activities of MCR 363 and BCOV. The pain suppression at the second phase therefore indicates the antihyperalgesic activity of the leaves which is useful in controlling acute pains. NSAIDs when acting supraspinally reduce the pain at both the phases of the Acetic acid test which was also observed in this experiment with MCR 363 and BCOV as well as with. These observations provide evidence that out of 126 synthetic compounds and 10 herbal drugs only few possess some antiinflammaroty activity e.g. MCR 363, BCOV, BNB, PME. The *in vitro* studies suggested that this anti-inflammatory activity is via inhibition of cyclooxygenase pathways. Further studies will be undertaken to correlate the pharmacological activities with the chemical constituents.

In Adjuvant induced arthritis model novel compound MCR 363, BCOV and herbal extracts BNB, PME resulted in more or less extent amelioration of the arthritis as effectively as Indomethacin. These results corresponded with that of Al-Haboubi and Zeitlin.[203] Indomethacin significantly reduced the footpad volume compared with the intact control on day 7 ($P<0.05$)(table 14). On the other hand, MCR 363 reduced the footpad volume as compared to vehicle treated adjuvant arthritis control group. Moreover, test compounds MCR 363, BCOV, BNB, PME did not modify the relative organ weight and body weight gain during the study period, whereas Indomethacin atrophied the weight of the thymus, spleen, and adrenals and significantly inhibited the body weight gain. This was done because the intensity of arthritis is influenced by the methods of

immunization of adjuvant. Strong bone loss after intense arthritis is induced when adjuvant is injected into the footpad, so this could be a model suitable for evaluating the bone resorption. However, the inflammation of the joint of this model was too strong, resulted in weight decreases in all groups including Indomethacin and test drugs MCR 363, BCOV, BNB, PME, If the adjuvant is injected into the base of the tail, a moderate degree of chronic arthritis is induced in the four limbs without causing a weight loss; it could be suitable for evaluating the inflammation of arthritis. The findings in this study suggest the use of MCR 363, BCOV, BNB, and PME may offer effective therapeutic approaches(Fig. 32,33,35).

The results obtained in the present study confirm the therapeutic efficacy of test drugs MCR 363, BCOV, BNB, PME when administered orally at doses 100,100,500,500 mg/kg/p.o. respectively the in the chronic stage of the TNBS model of colitis. MCR 363 was able to reduce macroscopic colonic damage, as scored according to the severity and extent of involved tissue, and also ameliorated the histological lesions that characterize this experimental model of colitis. BCOV at 100mg/kg/p.o. showed inhibition over the 18 days treatment. BNB and Pomogranate have also shown inhibition of colon damage from TNBS induced colitis. (Fig. 36)

It has been reported that inhibitors do not cause gastric lesions even at above effective anti-inflammatory doses[176,177] in contrast to NSAIDs, which induce gastrointestinal lesions after a single acute dose. The major side effect of conventional NSAIDs is gastropathy manifested as gastric bleeding, ulceration, alterations in gut motility, emesis and diarrhea, thought to be caused by their inhibition of COX-1 in the gastrointestinal tract. Indomethacin, at 20 at 30mg/kg/p.o. induced visible, dose-dependent hemorrhagic gastric lesions in rats 4 hr after dosing (Table-23, figure 36). In comparison MCR 363, BCOV, BNB, PME were without any damage to gastric mucosa at doses up to 100mg/kg/p.o. for synthetic compounds and 1000mg/kg/p.o. for herbal drugs. This contrasts strongly with current NSAIDs, in which case the major cause of withdrawal is gastrointestinal complications.

In the present study, the resting mean arterial blood pressure of urethane anaesthetized g. pigs was found to be 123±5 mmHg which is similar to the value reported for barbiturate anaesthetized rats[204]. Administration of 100 mg/kg/p.o. of the MCR 363 no significant difference found up to 3hrs as compared to vehicle treated control group. BCOV treatment with 100mg/kg/p.o. dose showed mean blood pressure 125±5 which was similar to control group. Neither BNB at 1000mg/kg dose nor PME at 1000mg/kg have any significant effect on mean blood pressure.

In vehicle treated control animals the heart rate was 350±21 and the QT interval 88±1 given in table 24. Test compounds MCR 363, BCOV, BNB and PME exhibit no significant effect on Heart rate and QT interval in G. pigs. The results of this study demonstrate that the anaesthetized guinea pig is an appropriate model for early assessment of cardiovascular safety pharmacology. Overall, our results on heart rate, blood pressure, and ECG with reference compounds show the usefulness of the guinea pig as an animal model for the assessment of cardiovascular safety figure37,38 and table 24 expressed that that acute treatment of test drugs MCR 363, BCOV, BNB and PME are quite safe special aspect to heart rate, mean blood pressure and ECG in guinea pigs.

Isolated aorta contraction by PE was significantly relaxed by MCR363 (figure 39,40), This is very important finding because relaxation of aorta effect is via NO releasing property of MCR 363, this property make this compounds cardio protective contrast to other COX-2 inhibitors. BCOV, BNB, PME have not shown any effect on isolated aortal response.

In the present work, the effects of test compounds MCR 363, BCOV, BNB, PME were studied in several behavior animal models, such as locomotor activity, behavioral tests, barbiturate-induced sleeping time and rota rod tests, to investigate its possible central activity. These tests are classical models for screening central nervous system actions providing information about psychomotor performance, anxiety, myorelaxant activity and depression. It is well known that benzodiazepines act as anxiolytics (at low doses), anticonvulsants, and also produce sedation and a myorelaxant effect at higher doses.[205] Thereby, our group has used diazepam at 1 mg/kg/p.o.. The results suggest that none of the test drugs MCR 363, BCOV, BNB, PME have any significant effect on CNS.

According to OECD-420 guideline and Ghosh,[206] Compounds MCR 363, BCOV BNB and PME extract can be classified as safer compounds, since the LD_{50} was found to be more than 5.0 g/Kg. The gram equivalent of the LD_{50} of the extract in an adult man would be more than 500g that is, a plate full of this test substance making it relatively safe. It has been observed that overdose of any of the above listed drugs is usually non-fatal; the animals tend to suffer self-limiting gastrointestinal disturbances. Likewise, the test substances were administered orally to the test animals. This way the same route used by the traditional healers in treating their patients was used in the test animals. This would make any findings in the mice easily translatable to what would be expected in the human subjects. The viscera of the animals did not show any macroscopic changes that could point to the cause of death. However, because of genetic variation in response to drugs by different species, it is difficult to directly translate the results of this study to other animal species or to man.

The purpose of this study was to look at the toxicity profile of the MCR 363, BCOV, BNB, and PME. A 28-days study is considered a subacute study , which is well accepted for eliciting any toxicity on long-term feeding. These test drugs MCR 363, BCOV, BNB, and PME did not appear to retard growth or affect food consumption and utilization (Tables 30,31). The feed conversion efficiency of the different groups followed a similar pattern indicating a normal metabolism of the animals. The mean food consumption of the animals in the control and experimental groups was similar (Tables 30). This finding indicates that the food intake and utilization of protein and other nutrients were not affected by the intake of the test drugs MCR 363, BCOV, BNB, and PME. Moreover, there were no differences between the genders with respect to feed con-version efficiency.
There were no significant changes in the hematological parameters between the control and the experimental groups (Tables 32), suggesting that the MCR 363, BCOV, BNB, PME may not be toxic as they do not affect the circulating red cells, nor the hematopoiesis and leucopoiesis that could otherwise have caused a megaloblastic anemia, nor changes in packed cell volume (PCV) and eosinophils. From gross pathology observations and different organ weights(Table 33) suggests that there was no toxic effect observed on vital

organs e.g. lung, liver, kidney, heart, brain, spleen, ovary and testis that indicates these compounds are relatively safe to use via oral route.

It is clear that the liver and kidneys play significant roles in various metabolic processes. Therefore, emphasis was placed on the effect these compounds might have on the function of these organs. In addition, the liver plays an important role in xenophobic function, while kidneys are sites for reabsorption. No Histopathological abnormality found in kidney and liver sample of rat fed with of MCR 363, BCOV, BNB, PME.

In our efforts to determine the possible use of MCR 363, BCOV, BNB, and PME we have evaluated the acute and subacute toxicity of these four test substances. Our findings indicate that these substances are nontoxic and well tolerated for the 28-day study period and therefore have potential for safe use.

These finding suggest that the compounds MCR 363, BCOV, BNB, and PME showed anti-inflammatory activity in both *in vitro* and *in vivo* models via possible mechanism of selective COX-2 inhibition and NO release effects.