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Synthesis, Characterization and Anti-Inflammatory Activity of Metal Complexes of 5-Methyl-N-[4-(Trifluoromethyl) Phenyl]-Isoxazole-4-Carboxamide on Carrageenan Induced Arthritic Rats

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- Metal complexes
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- Anti-inflammatory activity

Abstract

Leflunomide, a type of disease modifying antirheumatic drug (DMARD) is a pyrimidine synthesis inhibitor which is chemically and pharmacologically very heterogeneous. It was selected as a bioactive ligand to react with hydrated salts of essential and trace metals to afford $[M(Lef)_4]X_2$, type complexes which were characterized by FT-IR, ^{1}H-NMR and elemental analysis. In these reactions leflunomide acted as monodentate ligand coordinated to metal ions through a cyanide group.

The antiinflammatory activity of leflunomide and synthesized metal complexes were examined in carrageenan induced arthritic rats at two different doses i.e. 5 mg Kg^{-1} and 7.5 mg Kg^{-1}. Leflunomide showed 99.56±0.19 and 99.81±0.71 % edema inhibition at above mentioned doses, while in all leflunomide-metal complexes, the carrageenan induced anti-inflammatory activity of leflunomide decreased significantly ($p< 0.005$) indicating that metals affected leflunomide activity on complexation. These changes are in dose response relationship.

Maximum retention of leflunomide anti-inflammatory activity was observed in leflunomide-manganese complex. Results also indicated dose response relationship as leflunomide metal complexes of manganese, ferrous, ferric, copper and nickel showed reasonable retention of anti-inflammatory activity at 5mg Kg^{-1} but their activity reduced markedly at 7.5mg Kg^{-1}. In metal complex of calcium, chromium, cobalt and zinc anti-inflammatory activity increased as the dose increased.

INTRODUCTION

Leflunomide (Figure 1), 5-methyl-N-[4-(trifluoromethyl) phenyl]-isoxazole-4-carboxamide [1-2] is a leading disease modifying antirheumatic drug to treat rheumatoid arthritis (RA). This drug contains isoxazole ring system. Pervious studies indicated that isoxazole group acts as monodentate ligand [3-6]. During chelation metals coordinate with isoxazole system via one metal ion [5] and some times three and six [4]; studies also indicated that inside human body ferrous metal of cytochrome P450 coordinates with isoxazole ring nitrogen or oxygen present in leflunomide either by charge transfer or by cleaving the N–O bond of isoxazole ring via deprotonation of the carbon attached to isoxazole nitrogen [7]. RA patients usually administer many essential and trace metals. It was mentioned that these patients usually take NSAIDs [8] and they generally develop stomach hyperacidity problem due to prostaglandin inhibition (mainly prostaglandin E) [9,10]. To overcome this situation, they habitually take antacids that contain hydroxides of magnesium, aluminum and calcium. Numerous studies highlighted that multivalent cations, which may either be present in low concentrations in human body or may be ingested as a result of multiple drug therapy, reduce the absorption of other drugs [11-14].

We have already reported in-vitro interactions of leflunomide with many metals of biological interest [15]. Although *in vitro* studies regarding these interactions may provide a preliminary

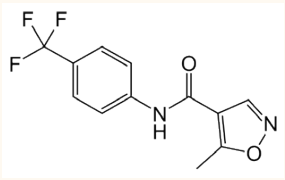

Figure 1 Structure of leflunomide.

data but *in vivo* studies especially on animals provide complete knowledge of drug behavior when it enters to a complex living system. Regarding *in vivo* study of DMARDs that possess analgesic and anti-inflammatory property are evaluated on the basis of their ability to suppress inflammation and relieve the symptoms. For this purpose these agents are not directly evaluated on human subjects because of the possible side effects and unwanted interaction; hence *in vivo* preclinical studies are usually carried out. This work is in continuation of our previous work of synthesis of drug metal complexes [16- 19] and drug-metal interactions [20-23].

The aim of this study was to synthesize leflunomide metal complexes, to study their complexation behavior and to observe their antiinflammatory effect on carrageenan induced arthritic rats which represent an acute inflammatory animal model.

EXPERIMENTAL

Material and reagents

All the chemicals used were of analytical grade and of highest purity. Leflunomide (reference standard) was gratis by Hilton Pharma. The essential and trace elements used in the form of their hydrated salts as magnesium chloride ($MgCl_2.6H_2O$), calcium chloride ($CaCl_2.2H_2O$), chromium chloride ($CrCl_3.6H_2O$), manganese chloride ($MnCl_2.4H_2O$), ferric chloride ($FeCl_3.6H_2O$), ferrous Sulfate ($FeSO_4.7H_2O$), cobalt chloride ($CoCl_2.6H_2O$), nickel chloride ($NiCl_2.6H_2O$), copper chloride ($CuCl_2.2H_2O$), zinc chloride ($ZnCl_2$) and cadmium chloride ($CdCl_2.H_2O$) were of analytical grade. Solvents include ethanol, methanol, dimethylsulfoxide (DMSO) used were from TEDIA (USA) were purchased from local dealers.

Instrumentation

FT-IR instrument was Shimadzu model Prestige-21 spectrophotometer connected to a PIV computer loaded with Shimadzu IR solution-1.2 software. Samples in the form of KBr pellets were scanned in the region of 400-4000 cm^{-1}. Proton NMR studies were carried out on a Bruker AMX 500 MHz spectrometer in deuterated water, methanol and chloroform using TMS as an internal standard. Carbon, hydrogen and nitrogen (CHN) elemental studies were carried out using a Perkin-Elmer CHN 2400.

UV visible spectrophotometer (Model 1601, Shimadzu, Japan) with 10-mm path length connected to a P-IV computer loaded with Shimadzu UVPC version 3.9 software and 1 cm rectangular quartz cells, were used in Job's studies. Digital Gallenkamp was used to determine melting point.

Jobs studies

The stoichiometries of the complexes were determined by Job's method of continuous variation at 37 °C [24-26] before synthesizing leflunomide-metal complexes. For this purpose alcoholic solutions of leflunomide and metal salts (equi-molar concentrations) were prepared individually and mixed in different ratios from 9:1, 8:2 to 1:9 keeping the final volume 10 ml. Solutions were kept at 37 °C for half an hour and then analyzed in the region 200-800 nm by UV/visible spectrophotometry and the maxima recorded.

Synthesis of leflunomide-metal complexes

After getting evidence of interaction and determination of stoichiometric ratios from Job's studies [26], metal complexes of leflunomide were synthesized in the ratio of 4:1(ligand: metal). Hydrated salt solution (in methanol) of each metal was individually added to the methanolic solution of leflunomide and refluxed for 2-3 hours at 80°C with constant stirring and then filtered; filtrate was left for slow crystallization at room temperature and the product obtained was dried. Purity of all synthesized complexes was checked by TLC on pre-coated silica gel plates using methanol/ethyl acetate as eluting solvent in different ratios (1:1/1:2 v/v) and spots were detected under UV lamp. The compounds were then recrystallized from the same solvent. Melting points and solubility were recorded and then characterized by means of spectroscopic techniques involving IR, ^{1}H-NMR and CHN elemental analysis.

Anti-inflammatory studies

Animals: Female Sprague–Dawley rats, weighing 215–230 g (8–10 weeks), kept at 21 ± 2°C on a 12-hour light/dark cycle with free access to standard laboratory rat food pellets and water, were used for this study under the ethical guidelines of International Association for the Study of Pain in conscious animals [27].

Treatment protocol: Rats were randomly distributed (n=6) into ten different groups as shown in table 5. Each group received their respective therapy one hour prior to arthritis induction. All drugs were given via oral route using 0.5ml dimethyl sulfoxide (DMSO) as vehicle.

Effect of leflunomide and its metal complexes were studied on carrageenen induced acute arthritic model at two different doses (5mg kg^{-1} and 7.5mg kg^{-1}) to study dose-response relationship.

In-vivo experimental design

Induction of arthritis: After one *hour* of the treatment, arthritis was induced in all animals. For this purpose, acute model of arthritis i.e. arthritis induced by carrageenan was selected. Arthritis was induced by injecting 0.1 mL of 1% carrageenan solution in normal saline through intra-dermal route at rat's right paw. This time was measured as zero.

Clinical assessment of arthritis: Arthritic severity was evaluated from time '0' up to five hour (as during this period the group who received leflunomide was fully recovered) by determining change in the right paw volume. This change was determined by water displacement method using plethysmometer (model 7140; Ugo Basile, Varese, Italy). This instrument has

capability to measure paw tibiotarsal joint in three dimensions. Thus any variability of the pattern of swelling of individual limbs can be monitored.

Statistical analysis

Edema inhibition (%) was calculated for every hour, using the formula Edema rate E % = $V_T - V_0 \backslash V_0 * 100$, Where, E % edema rate (%), V_0 is volume of rat's hind paw before 1% carrageenan administration and V_T is volume of rat's hind paw at t hour, while percentage inhibition was calculated by I % = $E_C - E_T \backslash E_C * 100$, where, E_C = Edema rate of control group and E_T = Edema rate of test compound at t hour.

Data was analyzed by using one way analysis of variance using statistical package for social sciences software (SPSS INC). Dunnet's post-hoc test was conducted to determine group mean differences taking significant level $p<0.05$ and $p<0.005$ highly significant.

RESULTS AND DISCUSSION

RA is a progressive inflammatory disease of unknown etiology that causes severe disability and increases mortality [28,29]. Early use of DMARDs has become the standard for its treatment. However, an incomplete response to DMARD monotherapy is observed in some patients [30 – 32]. Leflunomide an immunomodulatory drug inhibits mitochondrial enzyme dihydroorotate dehydrogenase (abbreviated as DHODH) is involved in the synthesis of the pyrimidine ribonucleotide uridine monophosphate (rUMP) [33], this inhibition of human DHODH by A77 1726, the active metabolite of leflunomide, occurs at levels (approximately 600 nM) that are achieved during treatment of rheumatoid arthritis (RA) [34].

Job's studies

In order to determine maximum complexation point where leflunomide interacts with metals, Job's method of continuous variation was executed. The ligand substrate ratios were evaluated from the graphs (Figures 2a & 2b) and it was found that leflunomide interacted with all the above mentioned metals in the ratio of 4:1.

Synthesis of leflunomide-metal complexes

Lefunomide metal complexes were synthsized by refluxing leflunomide and essential and trace elements for 2-3 hours at 80 °C in the methanolic solutions followed by usual workup. These complexes were insoluble in benzene, chloroform and water but soluble in methanol and DMSO. Physical characteristics of these are given in Table 1.

For the characterization of these complexes, different techniques as IR, ^{1}H-NMR and CHN elemental analysis were applied. There are a number of free coordinating sites present

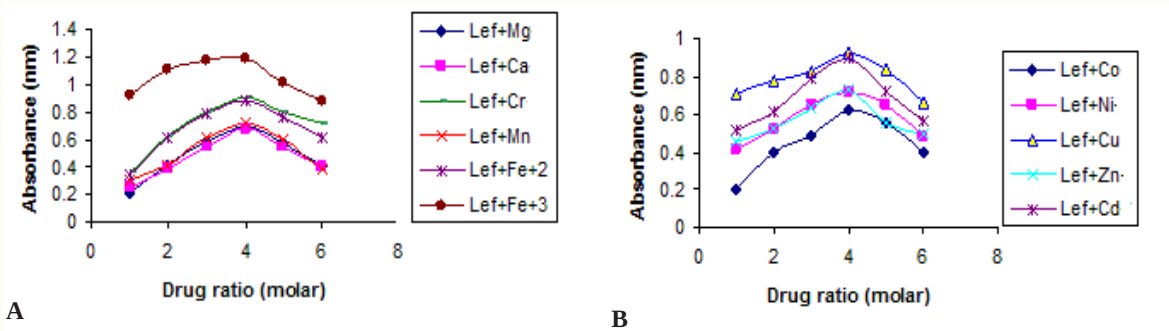

Figure 2 Jobs plot of leflunomide-metal complexes.

Table 1: Physical characteristics of leflunomide and its metal complexes.

S.No	Compound	Color	M.P	% Yield
1	Leflunomide	White	166	----
2	Lef+Mg	white	146	65
3	Lef+Ca	white	125	75
4	Lef+Cr	white	160	81
5	Lef+Mn	peach	172	76
6	Lef+Fe^{+2}	faun	121	69
7	Lef+Fe^{+3}	light yellow	152	85
8	Lef+Co	light purple	148	74
9	Lef+Ni	light geen	154	88
10	Lef+Cu	green	158	89
11	Lef+Zn	white	153	79
12	Lef+Cd	white	141	85

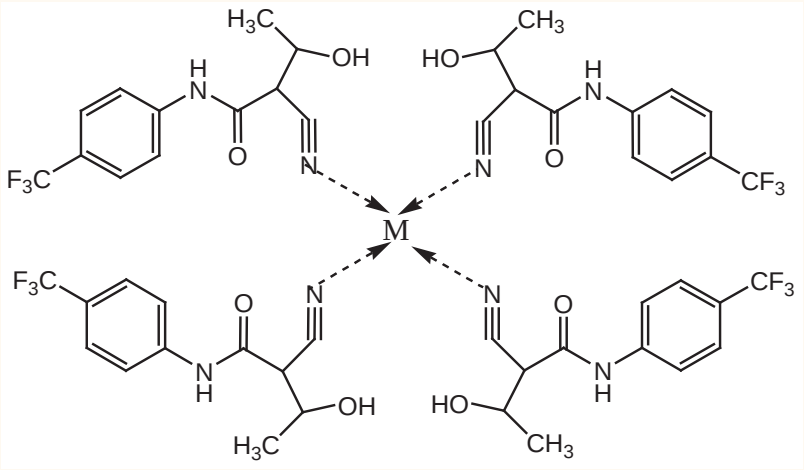

Figure 3 Proposed structure of leflunomide-metal complexes.

in the isoxazolyl moiety of leflunomide molecule. Among them, the nitrogen or oxygen atoms present in isoxazole ring become very important with regard to drug-metal interaction. Previous studies indicated that inside human body ferrous metal of cytochrome P450 co-ordinates with isoxazole ring nitrogen or oxygen present in leflunomide either by charge transfer or by cleaving the N–O bond of isoxazole ring via deprotonation of the carbon attached to nitrogen present in isoxazole ring [7].

Infrared spectroscopy

Presence of numerous functional groups in leflunomide molecule makes its infrared (IR) spectrum quite complicated. In IR spectra of leflunomide (Table 2) a clear sharp characteristic peak at 3358cm^{-1} was attributed to NH peak of amide. A sharp doublet peak appearing at 2924 cm^{-1} in spectrum was assigned to CH stretching vibration. HC=N-O sharp peak present in isoxazole ring appeared at 1690cm^{-1} in leflunomide spectra. The IR spectrum of leflunomide showed sharp peak at 1604cm^{-1} which was assigned to C=O of amide while peak at 1504cm^{-1} was attributed to C=C.

On complexation of leflunomide with metals, the intensity of characteristic NH peak at 3358cm^{-1} merged with that of hydroxyl (OH) appearing with high intensity in the range 3425 - 3444 cm^{-1}. A small shifting (2926- 3120cm^{-1}) has been observed in CH peak in case of leflunomide-metal complexes whose intensity is reduced in all complexes except in iron and manganese complexes while in case of leflunomide Cu complex, intensity of CH peak remains normal as that of parent drug. In all leflunomide metal complexes, a new sharp and intense peak in the range 2358 - 2394 cm^{-1} appeared which was attributed to CN stretching. The intensity of this peak was medium in all metal complexes except Mn, Ni and Zn where it became more intense. The C=O of amide and C=C peaks are slightly shifted from 1603 to 1610 cm^{-1} and 1504 -1556 cm^{-1} upon bonding respectively with the reduction of intensity in case of all metal complexes excluding iron and cadmium.

By comparing IR spectra of leflunomide with its metal complexes, major conclusion can be drawn as appearance of OH and CN peak in all leflunomide-metal complexes indicating the opening of isoxazole ring which was absent in IR of leflunomide. These points confirm that refluxing of leflunomide with metals opens the isoxazole ring of the drug with the formation of cyanide (CN) group where a peak at 2358 - 2394 cm^{-1} was observed, this shifting indicates the point of attachment which was supported by the previous study that cyanide is negatively charged and has high potency to bind with many metals outside [47] and inside the living body [35-37].

NMR spectroscopy

The ^{1}H NMR spectra (Table 4) of parent molecule showed a singlet at δ: 2.49 ppm corresponding to CH_3 protons attached to the isoxazole ring; two doublets at δ: 7.55 ppm at δ: 7.270 ppm for CH of phenyl ring system; singlet at δ: 8.64 ppm corresponding to NH peak of amide group.

The ^{1}H-NMR spectra of leflunomide was compared with its metal complexes. These spectra showed a set of signals which were almost identical to those of parent molecule, except a signal corresponding to CH_3 group that showed shifting of peaks in the region δ 1.2-1.67 ppm. This change in the ^{1}H-NMR spectra of the complexes was due to involvement of cyanide coordination of the drug to the metal (Table 3).

Elemental analysis

The results obtained from elemental analysis CHN, indicate that all of the isolated complexes are formed from the reaction of the drug with metal salt in 4:1 molar ratio as shown in Table 4.

Anti-inflammatory studies of leflunomide-metal complexes: The evidence of clinical tenderness and erythmia was observed from 30 minutes showing erythmia in ankle, metatarsal and interphalangeal joints in all inflammatory groups (Tables 6 and 7). But after 30 minutes data of percent inhibition showed significant ($p < 0.005$) affect of treatment at both doses throughout the experiment. Results also pointed out the dose response relationship in leflunomide-metal complexes. Variation was observed in anti-inflammatory activity in all leflunomide-metal complexes through out the experiment at both doses when compared with CIA-lef (carrageenan induced arthritic group received leflunomide).

Table 2: FT-IR absorption data of leflunomide and its metal complexes (4000-400 cm^{-1}).

S.No	Drug	NH	OH	CH	C=N-O	CN	C=O	C=C
1	Lef	3358(d)		2924(d)	1690(s)		1604(s)	1504(s)
2	Lef+Mg	3355(d)	3444(w)	2950(d)		2358s	1604(s)	1523(s)
3	Lef+Ca	3358(d)	3453(w)	2933(d)		2362.5(s)	1607(s)	1528(s)
4	Lef+Cr	3356(d)	3442.94(w)	2931(d)		2390(s)	1608(s)	1556(s)
5	Lef+Mn	3348(d)	3437(w)	2931(d)		2362.8(s)	1608(s)	1539(s)
6	Lef+Fe^{+2}	3348(s)	3442.94(w)	2926(d)		2398(s)	1604(s)	1544(s)
7	Lef+Fe^{+3}	3359(d)	3437(w)	2926(s)		2368(s)	1603(s)	1556(s)
8	Lef+Co	3358(s)	3425(w)	2926(d)		2374(s)	1608(s)	1539(s)
9	Lef+Ni	3368(s)	3442(w)	2931(d)		2380(s)	1604(s)	1550(s)
10	Lef+Cu	3338(s)	3442.94(w)	2931.8(d)		2394(s)	1603(s)	1556(s)
11	Lef+Zn	3354(d)	3442.94(w)	2926(d)		2389(s)	1608(s)	1550(s)
12	Lef+Cd	3333(s)	3441.2(w)	3120(d)		2365(s)	1608(s)	1543(s)

s= singlet, d= doublet, w=wide

Table 3: H-NMR data of leflunomide and its metal complexes.

S.No	Drug	H-NMRδ: ppm
1	Lef	8.64(1H, bs,NH), 7.5-7.9 (4H,bs,phenyl), 2.49 (3H,s, CH_3)
2	Lef+Mg	8.66(1H, bs,NH), 7.6-7.68 (4H,bs,phenyl), 1.64(3H,s, CH_3)
3	Lef+Ca	8.56(1H, bs,NH), 7.5-7.68 (4H,bs,phenyl), 1.68(3H,s, CH_3)
4	Lef+Cr	8.45(1H, bs,NH), 7.6-7.7 (4H,bs,phenyl),1.54(3H,s, CH_3)
5	Lef+Mn	8.45(1H, bs,NH), 7.5-7.7 (4H,bs,phenyl), 1.57(3H,s, CH_3)
6	Lef+Fe^{+2}	8.46(1H, bs,NH), 7.5-7.69 (4H,bs,phenyl),1.57(3H,s, CH_3)
7	Lef+Fe^{+3}	8.45(1H, bs,NH), 7.6-7.7 (4H,bs,phenyl), 1.2(3H,s, CH_3)
8	Lef+Co	8.47(1H, bs,NH), 7.58-7.68 (4H,bs,phenyl), 1.6(3H,s, CH_3)
9	Lef+Ni	8.45(1H, bs,NH), 7.48-7.69 (4H,bs,phenyl), 1.5(3H,s, CH_3)
10	Lef+Cu	8.46(1H, bs,NH), 7.5-7.7 (4H,bs,phenyl), 1.59(3H,s, CH_3)
11	Lef+Zn	8.66(1H, bs,NH), 7.5-7.7 (4H,bs,phenyl), 1.2(3H,s, CH_3)
12	Lef+Cd	8.46(1H, bs,NH), 7.5-7.6 (4H,bs,phenyl), 1.6(3H,s, CH_3)

Table 4: Elemental analysis of leflunomide and its metal complexes.

S.No	Compound	C %	H %	N %
1	Lef	53.44(53.34)	3.27(3.36)	10.32(10.37)
2	$[Mg(Lef)_4]Cl_2$	46.21(46.19)	3.52(3.55)	8.77(8.98)
3	$[Ca(Lef)_4]Cl_2$	47.03(46.95)	3.30(3.28)	9.15(9.13)
4	$[Cr(Lef)_4]Cl_2$	46.53(46.50)	3.27(3.25)	9.08(9.04)
5	$[Mn(Lef)_4Cl_2$	47.03(47.07)	3.09(3.13)	9.05(9.15)
6	$[Fe^{+2}(Lef)_4]SO_4$	41.20(41.15)	3.21(3.17)	8.03(8.01)
7	$[Fe^{+3}(Lef)_4]Cl_3$	44.01(43.82)	3.71(3.68)	8.55(8.52)
8	$[Co(Lef)_4]Cl_2$	46.19(46.24)	3.17(3.23)	8.91(8.99)
9	$[Ni(Lef)_4]Cl_2$	45.01(44.95)	3.49(3.46)	8.82(8.74)
10	$[Cu(Lef)_4]Cl_2$	43.35(43.30)	4.28(4.24)	8.37(8.32)
11	$[Zn(Lef)_4]Cl_2$	45.66(45.71)	3.81(3.84)	8.72(8.88)
12	$[Cd(Lef)_4]Cl_2$	43.02(42.89)	4.01(3.90)	8.49(8.34)

Calculated values are given within parenthesis

Table 5: Group treatment.

S.No	Groups	Treatment
1	CIA control	No treatment arthritic induced rats
2	CIA lef	Leflunomide (Lef)
3	CIA lef+Mg	Lef -magnesium complex
4	CIA lef+Ca	Lef –calcium complex
5	CIA lef+Cr	Lef -chromium complex
6	CIA lef+Mn	Lef -magenese complex
7	CIA lef+Fe^{+2}	Lef -ferrous complex
8	CIA lef+Fe^{+3}	Lef -ferric complex
9	CIA lef+Co	Lef -cobolt complex
10	CIA lef+Ni	Lef -nickle complex
11	CIA lef+Cu	Lef -cupper complex
12	CIA lef+Zn	Lef -zinc complex
13	CIA lef+Cd	Lef -cadmium complex

Table 6: Percentage inhibition at 5mg Kg^{-1}.

Groups	Time(hours)				
	1	2	3	4	5
CIA lef	49.5±0.08	82.56±0.53	93.72±0.49	98.78±0.13	99.56±0.19
CIA lef+Mg	1.15±0.061**	3.42±0.05**	10.4±0.73**	18.9±0.83**	26.1±0.047**
CIA lef+Ca	2.5±0.21**	6.47±0.35**	15.38±0.07**	22.91±0.15**	30.16±0.46**
CIA lef+Cr	51.85±0.51**	33.16±0.26**	34.17±0.48**	65.29±0.16**	30.46±0.41**
CIA lef+Mn	6 9.48±0.11**	38.28±0.25**	63.6±0.61**	75.62±0.72**	96.6±0.26*
CIA lef+Fe^{+2}	5.38±0.015**	39.5±0.27**	32.74±0.53**	52.95±0.41**	84.96±0.52**
CIA lef+Fe^{+3}	21.59±0.15**	79.43±0.021**	48.23±0.41**	78.5±0.019**	77.87±0.55**
CIA lef+Co	88.32±0.253**	65.35±0.14**	12.25±0.32**	34.56±0.52**	31.6±0.45**
CIA lef+Ni	6.1±0.05**	14.01±0.13**	11.48±0.54**	61.68±0.48**	58.7±0.13**
CIA lef+Cu	79.53±0.23**	86.93±0.016**	98.12±0.71**	29.2±0.091**	66.0±0.015**
CIA lef+Zn	1.14±0.003**	7.24±0.015**	11.5±0.048**	13.24±0.61**	25.1±0.079**
CIA lef+Cd	80.44±0.05**	62.75±0.15**	53.98±0.09**	61.85±0.15**	85.83±0.34**
One Way ANOVA (df= 10,22)	F_1=3804.009 p<0.005	F_2=1558.289 P<0.005	F_3=2543.249 p<0.005	F_4=2292.363 p<0.005	F_5=2614.2 p<0.005

Values are mean ± S.D.Significant difference by multiple comparision Dunnett t(2-sided) test*p<0.05, **p<0.005 from control and $^{+}$ p<0.05, $^{++}$ p<0.005 from CIA-lef.

Table 7: Percentage inhibition at 7.5mg Kg^{-1}.

Groups	Time(hours)				
	1	2	3	4	5
CIA lef	69.5±0.67	88.56±0.56	97.72±0.71	98.24±0.32	99.81±0.71
CIA lef+Mg	3.16±0.84**	4.52±.09**	7.89±.091**	14.17±0.57**	21.83±0.64**
CIA lef+Ca	4.2±0.45**	9.8±0.66**	17.6±0.59**	29.4±0.77**	42.1±0.68**
CIA lef+Cr	17.82±0.59**	52.2±0.61**	5.66±0.33**	45.26±0.32**	34.51±0.36**
CIA lef+Mn	39.36±0.94**	33.1±0.72**	23.12±0.47**	46.54±0.64**	51.77±0.46**
CIA lef+Fe^{+2}	20.69±0.37**	12.02±0.41**	2.02±0.91**	3.54±0.61**	36.01±0.93**
CIA lef+Fe^{+3}	53.43±0.61**	3.67±0.83**	18.56±0.82**	25.06±0.49**	46.1±0.86**
CIA lef+Co	12.89±0.53**	15.43±0.64**	8.32±0.38**	7.8±0.38**	4.41±0.53**
CIA lef+Ni	76.25±0.16**	3.2±0.48**	25.63±0.31**	27.58±0.91**	37.08±0.38**
CIA lef+Cu	67.9±0.61**	45.8±0.16**	27.7±0.42**	23.9±0.47**	46.9±0.45**
CIA lef+Zn	86.1±0.39**	39.52±0.82**	46.76±0.73**	16.54±0.55**	78.79±0.63**
CIA lef+Cd	80.46±0.46	90.47±0.92**	63.1±0.58	85.11±0.81**	83.46±0.24*
One Way ANOVA (df= 10,22)	F_1=1751.867 p<0.005	F_2=2128.241 p<0.005	F_3=2354.91 p<0.005	F_4=2251.116 p<0.005	F_5=5789.99 p<0.005

Values are mean ± S.D.Significant difference by multiple comparision Dunnett t(2-sided) test*p<0.05, **p<0.005 from control and $^{+}$ p<0.05, $^{++}$ p<0.005 from CIA-lef.

It has been reported earlier that leflunomide showed good anti-inflammatory property by inhibiting proinflammatory cytokines through DHODH (dihydroorotate dehydrogenase) inhibition [38]. Present results also showed the same consistency as leflunomide inhibited inflammation significantly ($p<0.005$). Results also indicated that in all leflunomide-metal complexes, the carrageenan induced anti-inflammatory activity of leflunomide altered significantly ($p< 0.005$) at both doses when compared with CIA-lefunomide, indicating that metals affected leflunomide activity on complexation.

By observing all the data, it can be concluded that among leflunomide-metal complexes, maximum retention of leflunomide anti-inflammatory activity was observed in lefunomide—manganese complex at the dose of 5 mg Kg^{-1} while in lefunomide -- cadmium complex only at the dose of 7.5mg Kg^{-1}. It has also been observed that reasonable retention of anti-inflammatory activity occurred by increasing the dose of leflunomide-metal complexes from 5mg Kg^{-1} to 7.5 mg Kg^{-1}, the activity reduced markedly as in case of complexes of manganese, iron (II), iron (III) , copper and nickle while not so as in leflunomide- cadmium complexe. Anti-inflammatory activity increased with the increase in dose in case of lefunomide -metal complexes with calcium, chromium, cobalt and zinc.

CONCLUSION

Leflunomide contains isoxazole ring system which opens during interaction with essential and trace metals. The cyanide group of isoxazole ring which acts as monodentate and involves in drug-metal complexation producing distorted square planar geometry. Variation was observed in anti-inflammatory activity in all leflunomide-metal complexes in comparision when leflunomide was given alone in carrageenan induced arthritic rats. These changes in activity indicated some dose response relationship.

Maximum retention of leflunomide anti-inflammatory activity was observed in leflunomide-magnese complex at the dose of 5 mg Kg^{-1} while in leflunomide-cadmium complex with the dose 7.5 mg Kg^{-1}. Results also indicated dose response relationship as the dose increased, as complexes of manganese, ferrous, ferric, copper and nickle showed reasonable retention of anti-inflammatory activity at 5mg Kg^{-1} but their activity reduced markedly at 7.5 mg Kg^{-1}. In metal complexes of calcium, chromium, cobalt and zinc anti-inflammatory activity increased with the increase of dose.

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