

Anti-oxidant, *in vitro*, *in vivo* anti-inflammatory activity and antiproliferative activity of mefenamic acid and its metal complexes with manganese(II), cobalt(II), nickel(II), copper(II) and zinc(II)

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Abstract

Some new complexes of mefenamic acid with potentially interesting biological activity are described. The complexes of mefenamic acid [Mn(mef)₂(H₂O)₂], **1**, [Co(mef)₂(H₂O)₂], **2**, [Ni(mef)₂(H₂O)₂], **3**, [Cu(mef)₂(H₂O)₂], **4** and [Zn(mef)₂], **5**, were prepared by the reaction of mefenamic acid, a potent anti-inflammatory drug with metal salts. Optical and infrared spectral data of these new complexes are reported. Monomeric six-coordinated species were isolated in the solid state for Mn(II), Ni(II) and Co(II), dimeric five-coordinated for Cu(II) and monomeric four-coordinated for Zn(II). In DMF or CHCl₃ solution the coordination number is retained and the coordinated molecules of water are replaced by solvent molecules. The anti-oxidant properties of the complexes were evaluated using the 1,1-diphenyl-2-picrylhydrazyl, DPPH, free radical scavenging assay. The scavenging activities of the complexes were measured and compared with those of the free drug and vitamin C. We have explored their ability to inhibit soybean lipoxygenase, β-glucuronidase and trypsin- induced proteolysis. The complex [Mn(mef)₂(H₂O)₂] exhibits the highest antioxidant activity and the highest inhibitory effect against the soybean lipoygenase (LOX), properties that are not demonstrated by mefenamic acid. Their inhibitory effects on rat paw edema induced by Carrageenan was studied and compared with those of mefenamic acid. The complex [Zn(mef)₂] exhibited a strong inhibitory effect at 0.1 mmol/Kg B.W. (81.5 ± 1.3% inhibition), superior to the inhibition induced by mefenamic acid at the same dose (61.5 ± 2.3% inhibition). Mefenamic acid and its metal complexes have been evaluated for antiproliferative activity *in vitro* against the cells of three human cancer cell lines: MCF-7 (human breast cancer cell line), T24 (bladder cancer cell line), A-549 (non-small cell lung carcinoma) and a mouse fibroblast L-929 cell line. The copper(II) complex displays against T24, MCF-7 and L-929 cancer cell lines, IC₅₀ values in a μM range similar to that of the antitumor drug *cis-platin* and they are considered for further stages of screening *in vitro* and/or *in vivo* as agents with potential antitumor activity.

Keywords: Mefenamic acid, NSAIDs, synthesis, metal complexes, antioxidant, LOX, *in vivo* anti-inflammatory, anti-proliferative activity

Introduction

Metal complexes with active drugs as ligands is a research area of increasing interest for inorganic and medicinal chemistry and has concentrated much attention as an approach to new drug development [1,2].

The synthesis of metal complexes with non-steroidal anti-inflammatory drugs (NSAIDs) as ligands has acquired new impetus in the past decade. First, on the basis of pure co-ordination chemistry NSAIDs are very versatile ligands and show a huge

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variety of ligating modes as function of the metal and the environmental conditions. The information collected from the preparative, structural and reactivity studies have high significance for several fields which span from the bio-sciences to the material sciences. Second, NSAIDs have numerous applications as pharmaceutical agents. Third, for this type of drugs the complex formation with specific metals may improve the activity towards certain diseases and hopefully may increase the activity spectrum. The combination of two or more different species into the same compound may bring to a multi-therapeutic agent which can be expanded by the synergic action of the metal residue once the coordination compound dissociate inside the target tissue. The coordination chemistry of non-steroidal anti-inflammatory drugs (NSAIDs) has been studied by several groups worldwide. Some complexes have increased pharmaceutical or biological activity with respect to the drug, or are interesting from a purely chemical point of view [3–6].

Most of the NSAIDs, including aspirin, possess a carboxylate group, which is able to coordinate metal ions. Non-steroidal anti-inflammatory drugs, NSAIDs, from the carboxylic acid family, derivatives of N-phenylanthranilic acid, such as mefenamic acid, tolfenamic acid and sodium diclofenac are widely used in inflammatory and painful diseases of rheumatic and non-rheumatic origin. The anti-inflammatory activity of NSAIDs and most of its

other pharmacological effects are related to the inhibition of the conversion of arachidonic acid, AA, to prostaglandins, which are mediators of the inflammatory process. The enzymes cyclooxygenase, COX and lipoxygenase, LOX, which catalyze the oxidative metabolism of AA, are useful targets for the design and the development of new drugs that substantially inhibit the generation of the final inflammatory products and the propagation of inflammation [7].

Mefenamic acid is a commonly used NSAID that is a cyclooxygenase-1 and 2 inhibitor with anti-inflammatory properties. It was found to exert neuroprotective effects and improve cognitive impairment *in vitro* and *in vivo* Alzheimer's disease models and also neuroprotective activities against neurodegeneration [8].

We have prepared some novel complexes of mefenamic acid, Figure 1, with Mn(II), Co(II), Ni(II), Cu(II) and Zn(II), $[Mn(mef)_2(H_2O)_2]$, $[Co(mef)_2(H_2O)_2]$, $[Ni(mef)_2(H_2O)_2]$, $[Cu(mef)_2(H_2O)_2] \cdot 2H_2O$ and $[Zn(mef)_2]$, in order to obtain information on structure-activity relationships for systems involving metal atoms. Crystal structures of dimeric tetraorganodistannoxane and monomeric triphenyl adducts of mefenamic acid have been already reported by our group [6]. The crystal structures of mefenamic acid and a copper(II) complex, $[Cu(mef)_2DMSO]_2$ have been solved, Figure 2 [10].

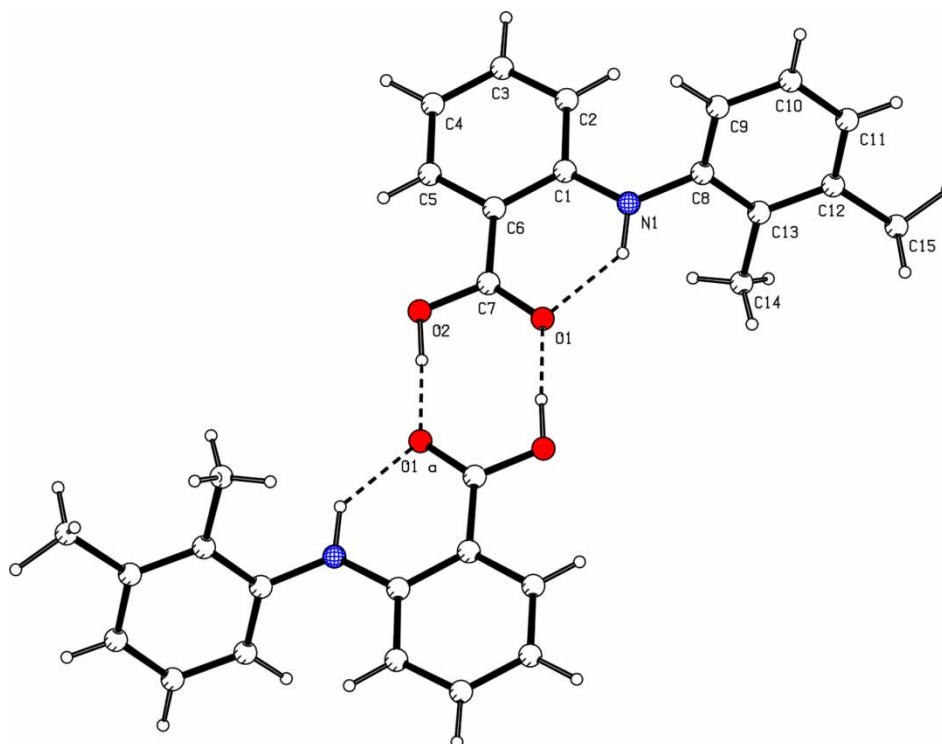


Figure 1. The structure of Hmef^{10a}.

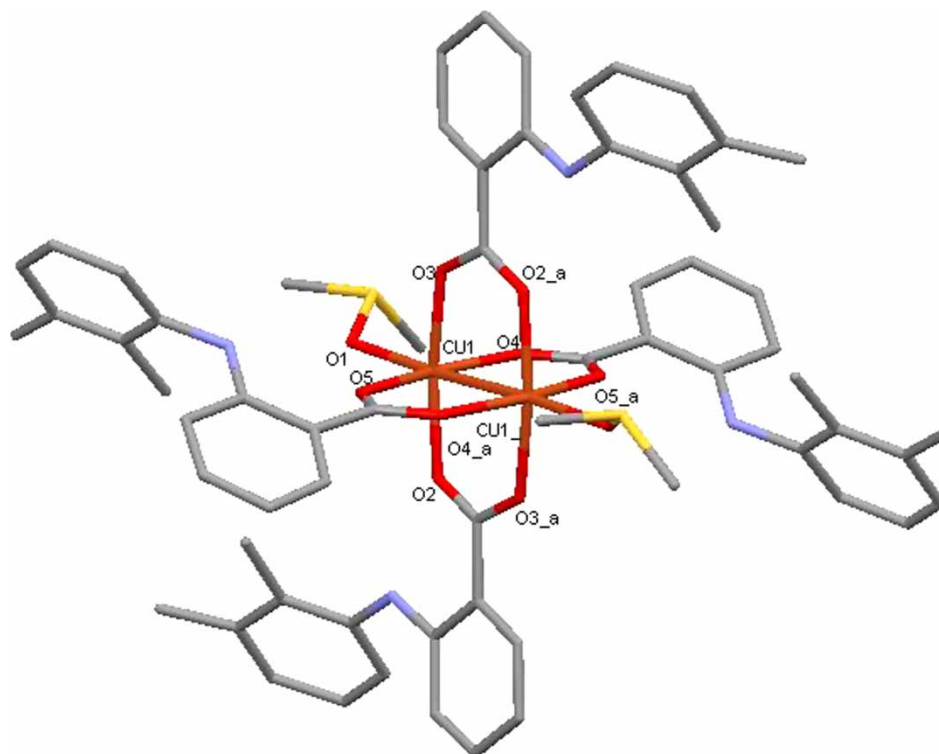


Figure 2. The structure of $[\text{Cu}(\text{mef})_2(\text{DMSO})]_2^{0b}$.

Here mefenamic acid and its metal complexes were tested for their antioxidant and anti-inflammatory activities in the rat carrageenin paw edema assay, a model for acute inflammation. Their inhibitory activities on LOX, on β -glucuronidase and on trypsin-induced proteolysis were evaluated.

It has been found that mefenamic acid induces apoptosis in human liver cancer cell-lines through caspase-3 pathway [9a]. Several NSAIDs, such as mefenamic acid, sulindac or indomethacin have been used in combination with a number of cytotoxic drugs cyclophosphamide, melphalanor and carmustine [9b]. The effect on cytotoxicity of clinically important NSAIDs drugs with a variety of chemotherapeutics was studied in different human cancer cells. A specific group of NSAIDs, indomethacin, sulindac, tolmetin, acemetacin, zomepirac and mefenamic, all at non-toxic levels, significantly increased the cytotoxicity of the anthracyclines, doxorubicin, daunorubicin and epirubicin, as well as teniposide, VP-16 and vincristine [9c]. A triphenyltin complex of mefenamic acid was tested for antimycobacterial activity against Mycobacterium tuberculosis H37Rv and was found to be a very good anti-tuberculosis agent [6b]. Here the cytotoxic activities of mefenamic acid and its metal complexes have been evaluated for antiproliferative activity *in vitro* against the cells of three human cancer cell lines: MCF-7 (human breast cancer cell line), T24 (bladder cancer cell line), A-549 (non-small cell lung

carcinoma) and a mouse L-929 (a fibroblast-like cell line cloned from strain L).

Herein, the goal is to define the probability to extend the pharmacological profile of mefenamic acid, in order to discover new properties such as antioxidant and anti-cancer activity and to prepare new complexes of mefenamic acid with essential metal ions, which would exhibit improved or different biological behaviour compared to the "parent drug", mefenamic acid.

Materials and methods

The reagents (Aldrich, Merck) were used as supplied while the solvents were purified according to standard procedures. Mefenamic acid was a gift from "VIA-NNEX. A.E". Trypsin (pancreas protease) 200Fip U/g, salicylic acid (SA), N-tosyl-methyl-arginine ester (TAME) 1,1-diphenyl-2-picrylhydrazyl (DPPH), nordihydroguaiaretic acid (NDGA) were purchased from the Aldrich Chemical Co. Milwaukee, WI, (USA). Soybean Lipoxygenase, linoleic acid sodium salt was obtained from Sigma Chemical, Co. (St. Louis, MO, USA) and carrageenin, type K, commercially available.

Melting points were determined in open capillaries and are uncorrected. C, H, and N analyses were carried out by the microanalytical service of the University of Ioannina. Infrared and far-infrared spectra were recorded on a Nicolet 55XC Fourier

transform spectrophotometer using KBr pellets ($4000-400\text{ cm}^{-1}$) and nujol mulls dispersed between polyethylene disks ($400-40\text{ cm}^{-1}$). UV spectra were acquired with a JASCO V-570 spectrophotometer UV/VIS/NIR.

Synthesis of $[Mn(mef)_2(H_2O)_2]$ (1)

A solution of $MnCl_2$ (0.0314 g, 0.26 mM) in methanol (3.0 mL) was added to a solution of mefenamic acid (0.1253 g, 0.52 mM) in methanol (1.5 mL). Drops of triethylamin, $N(Eth)_3$, were added till the apparent pH value was ~ 7 . The reaction mixture was stirred at room temperature for 1 h and cooled to 5°C in a refrigerator for 4 h. After the addition of a few drops of distilled water a pale brown powder was precipitated. The precipitate was collected by filtration, washed with cold water and dried in vacuo to afford (1). $[Mn(mef)_2(H_2O)_2]$ (1): D. p. 160°C . Yield 10%. IR (cm^{-1}): 3314m, $\nu(\text{NH})$; 3067m, 2942m $\nu(\text{CH}_3)$; 1612s $\nu_{\text{asym}}(\text{COO})$; 1503s $\nu_{\text{sym}}(\text{COO})$; 1579s $\nu_{\text{asym}}(\text{COO})$; 1456s $\nu_{\text{sym}}(\text{COO})$; 443ms $\nu(\text{Mn-O}_{\text{H}_2\text{O}})$; 374 mw, 245ms $\nu(\text{Mn-O}_{\text{oco}})$. $\mu_{\text{eff}} = 5.39\text{ MB}$. Anal. Calc. for $C_{30}H_{32}N_2O_6Mn$: C, 62.59; H, 5.27; N, 4.70. Found: C, 63.05; H, 5.64; N, 4.90; %.

Synthesis of $[Co(mef)_2(H_2O)_2]$ (2)

A solution of $Co(CH_3OO)_2 \cdot 4H_2O$ (0.0314 g, 0.125 mM) in methanol (1.5 mL) was added to a solution of mefenamic acid (0.0627 g, 0.26 mM) in methanol (1.5 mL). Drops of triethylamine, $N(Eth)_3$, were added till the apparent pH value was ~ 7 . The reaction mixture was stirred at room temperature for 1 h and cooled to 5°C in a refrigerator for 4 h. After the addition of a few drops of distilled water a violet powder was precipitated. The precipitate was collected by filtration, washed with cold water and dried in vacuo to afford (2). $[Co(mef)_2(H_2O)_2]$ (2): D. p. 115°C . Yield 80%. IR (cm^{-1}): 3552s, 3311s $\nu(\text{NH})$; 3062m, 2943m; $\nu(\text{CH}_3)$; 1649s, $\delta(\text{NH})$; 1613s, $\nu_{\text{asym}}(\text{COO})$; 1505s, $\nu_{\text{sym}}(\text{COO})$; 1579s, $\nu_{\text{asym}}(\text{COO})$; 1455mw, $\nu_{\text{sym}}(\text{COO})$; 429ms $\nu(\text{Co-O}_{\text{H}_2\text{O}})$; 408sh, 230ms $\nu(\text{Co-O}_{\text{oco}})$. $\mu_{\text{eff}} = 4.53\text{ MB}$. Anal. Calc. for $C_{30}H_{32}N_2O_6Co$: C, 62.62; H, 5.60; N, 4.87. Found: C, 63.07; H, 5.61; N, 4.92; %.

Synthesis of $[Ni(mef)_2(H_2O)_2]$ (3)

A solution of $NiCl_2 \cdot 6H_2O$ (0.0297 g, 0.125 mM) in methanol (1.5 mL) was added to a solution of mefenamic acid (0.0627 g, 0.26 mM) in methanol (1.5 mL). Drops of triethylamine $N(Eth)_3$ were added till the apparent pH value was ~ 7 . The reaction mixture was stirred at room temperature for 1 h and a light green powder was precipitated. The solution was cooled to 5°C in a refrigerator for 4 h. The precipitate

was collected by filtration, washed with cold methanol and dried in vacuo to afford (3). $[Ni(mef)_2(H_2O)_2]$ (3): D. p. 270°C . Yield 45%. IR (cm^{-1}): 3400br, $\nu(\text{NH})$; 3067m 2943m; $\nu(\text{CH}_3)$; 1613s $\nu_{\text{asym}}(\text{COO})$; 1503, $\nu_{\text{sym}}(\text{COO})$; 1579s $\nu_{\text{asym}}(\text{COO})$; 1455 $\nu_{\text{sym}}(-\text{COO})$; 450m $\nu(\text{Ni-O}_{\text{H}_2\text{O}})$; 379mw, 252ms $\nu(\text{Ni-O}_{\text{oco}})$. $\mu_{\text{eff}} = 3.13\text{ MB}$. Anal. Calc. for $C_{30}H_{32}N_2O_6Co$: C, 62.64; H, 5.60; N, 4.87. Found: C, 62.07; H, 6.03; N, 5.02; %.

Synthesis of $[Cu(mef)_2(H_2O)]_2 \cdot 2H_2O$ (4)

A solution of $Cu(CH_3COO)_2 \cdot H_2O$ (0.02499 g, 0.25 mM) in methanol (2.0 mL) was added to a solution of mefenamic acid (0.1253 g, 0.52 mM) in methanol (2.0 mL). Drops of triethylamine, $N(Eth)_3$, were added till the apparent pH value was ~ 7 . The reaction mixture was stirred at room temperature for 1 h and a dark green powder was precipitated. The solution was cooled to 5°C in a refrigerator for 4 h. The precipitate was collected by filtration, washed with cold methanol and dried in vacuo to afford (4). $[Cu(mef)_2(H_2O)]_2 \cdot 2H_2O$ (4): D. p. 155°C . Yield 52%. IR (cm^{-1}): 3341s, $\nu(\text{NH})$; 3067m 2943m; $\nu(\text{CH}_3)$; 1616s $\nu_{\text{asym}}(\text{COO})$; 1455m $\nu_{\text{sym}}(\text{COO})$; 429m $\nu(\text{Cu-O}_{\text{H}_2\text{O}})$; 348m, 277ms $\nu(\text{Cu-O}_{\text{oco}})$. $\mu_{\text{eff}} = 2.02\text{ MB}$. Anal. Calc. for $C_{30}H_{34}N_2O_7Cu$: C, 60.25; H, 5.72; N, 4.68. Found: C, 60.22; H, 5.58; N, 4.25%.

Synthesis of $[Zn(mef)_2]$ (5)

A solution of $ZnCl_2$ (0.05 g, 0.37 mM) in methanol (3.0 mL) was added to a solution of mefenamic acid (0.169 g, 0.7 mM) in methanol (3.0 mL). Drops of a methanolic solution 1 N NaOH were added till the apparent pH value was ~ 7 . The reaction mixture was stirred at room temperature for 1 h. After the addition of a few drops of distilled water a yellow powder was precipitated. The solution was cooled to 5°C in a refrigerator for 4 h. The precipitate was collected by filtration, washed with cold methanol/ H_2O , 5:1 and dried in vacuo to afford (5). $[Zn(mef)_2]$ (5): D. p. 115°C . Yield 20%. IR (cm^{-1}): 3341s $\nu(\text{NH})$; 3068m, 2942m; $\nu(\text{CH}_3)$; 1614s $\nu_{\text{asym}}(\text{COO})$; 1503s $\nu_{\text{asym}}(\text{COO})$; 1579s $\nu_{\text{asym}}(\text{COO})$; 1461s $\nu_{\text{sym}}(\text{COO})$; 456ms, 434sh, $\nu(\text{M-O}_{\text{oco}})$. Anal. Calc. for $C_{30}H_{28}N_2O_4Zn$: C, 66.01; H, 5.17; N, 5.13. Found: C, 66.06; H, 5.30; N, 5.00; %.

Biological assays

In vitro and in vivo [11]. In the in vitro assays each experiment was performed at least in triplicate and the standard deviation of absorbance was less than 10% of the mean.

Determination of the reducing activity of the stable radical 1,1-diphenyl-picrylhydrazyl (DPPH) [11]. To a solution of DPPH (0.1 and 0.5 mM) in absolute ethanol an equal volume of the compounds dissolved in ethanol was added. As control, solution ethanol was used. The concentrations of the solutions of the compounds were 0.1 and 0.5 mM. After 20 and 60 min at room temperature the absorbance was recorded at 517 nm.

Competition of the tested compounds with DMSO for hydroxyl radicals [11]. The hydroxyl radicals generated by the Fe^{3+} /ascorbic acid system, were detected according to Nash, by the determination of formaldehyde produced from the oxidation of DMSO. The reaction mixture contained EDTA (0.1 mM), Fe^{3+} (167 μM), DMSO (0.1 and 6.6 mM) in phosphate buffer (50 mM, pH 7.4), the tested compounds (concentration 1 mM) and ascorbic acid (10 mM). After 30 min of incubation (37°C) the reaction was stopped with CCl_3COOH (17% w/v).

Soybean lipoxygenase inhibition study in vitro [11]. *In vitro* study was evaluated as reported previously. The tested compounds (0.033 and 0.333 mM) dissolved in ethanol were incubated at room temperature with sodium linoleate (0.1 mM) and 0.2 mL of enzyme solution soybean lipoxygenase, dissolved in 0.9% NaCl solution [250 u/mL]. The conversion of sodium linoleate to 13-hydroperoxylinoleic acid at 234 nm was recorded during 5 min and compared with the appropriate standard inhibitor (nordihydroguaiaretic acid 0.1 mM 83.7% inhibition; 1 mM 94.7% inhibition).

Inhibition on β -glucuronidase [11]. Compounds in acetate buffer (0.1 mM, pH 7.4) were tested against β -glucuronidase (0.1 mL of 1 U/mL) with 2.5 mM p-nitrophenyl- β -D-glucopyranosiduronic acid. After incubation at 37°C for 30 min, 2 mL of 0.5 N NaOH solution was added to the mixture and the absorbance of the mixture was measured at 410 nm.

Inhibition on proteolysis [11]. Tosyl arginine methyl ester (TAME) was used as substrate for trypsin. The reaction mixture consisted of 0.75 mL buffer (0.1 M tris-HCl, pH 7.8 in 50% methanol v/v) and 0.14 mL TAME (0.01 M in 50% v/v methanol). Compounds dissolved in 50% methanol were added (0.3 mM). The reaction was started by addition of 0.1 mL trypsin (1 mg/mL 0.001 N HCl). The increase in the absorbance at 256 nm was determined in the next 4 min.

Antiproliferative assay in vitro [12]

Compounds. Test solutions of the compounds tested (1 mg/ml) were prepared by dissolving the substance in 100 μL of DMSO completed with 900 μL of tissue culture medium. Afterwards, the tested compounds were diluted in culture medium to reach the final concentrations of 100, 10, 1 and 0.1 $\mu\text{g}/\text{mL}$. The solvent (DMSO) in the highest concentration used in test did not reveal any cytotoxic activity.

Cells. The established *in vitro* human cancer cell line MCF-7 (human breast cancer cell line), T24 (bladder cancer cell line), A-549 (non-small cell lung carcinoma) and a mouse L-929 (a fibroblast-like cell line cloned from strain L) were applied: The cell lines are maintained in the Cell Culture Collection of the University of Ioannina. Twenty-four hours before addition of the tested agents, the cells were plated in 96-well plates at a density of 10^4 cells per well. The T-24 and MCF-7 cells were cultured in the D-MEM (Modified Eagle's Medium) medium supplemented with 1% antibiotic and 10% fetal calf serum. L-929 cells were grown in Hepes-buffered RPMI 1640 medium supplemented with 10% fetal calf serum, penicillin (50 U/mL) and streptomycin (50 mg/mL). A-549 cells were grown in F-12K Ham's medium supplemented with 1% glutamine, 1% antibiotic/antimycotic, 2% NaHCO_3 and 10% fetal calf serum. The cell cultures were maintained at 37°C in a humid atmosphere saturated with 5% CO_2 . Cell number was counted by the Trypan blue dye exclusion method. MCF-7, L-929 and A-549 cells were determined by the sulforhodamine B assay [13], and T24 cells by the MTT assay [12].

SRB assay. The details of this technique were described by Skekhan et al. [13]. The cytotoxicity assay was performed after 72-h exposure of the cultured cells to varying concentrations (from 0.1 to 100 $\mu\text{g}/\text{mL}$) of the tested agents. The cells attached to the plastic were fixed by gently layering cold 50% trichloroacetic acid (TCA) on the top of the culture medium in each well. The plates were incubated at 4°C for 1 h and then washed five times with tap water. The background optical density was measured in the wells filled with culture medium, without the cells. The cellular material fixed with TCA was stained for 30 min with 0.4% sulforhodamine B dissolved in 1% acetic acid. Unbound dye was removed by rinsing with 1% acetic acid. The protein-bound dye was extracted with 10 mM unbuffered Tris base for determination of optical density (at 540 nm) in a computer-interfaced, 96-well microtiter plate.

MTT. This technique was applied for cytotoxicity screening against T-24 cells growing in suspension culture. An assay was performed after 72 h exposure to varying concentrations (from 0.1 to 100 $\mu\text{g}/\text{mL}$) of the tested agents. For the last 3-4 h of incubation

20 μ L of MTT solution was added to each well [MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma]; stock solution: 5 mg/mL]. The mitochondria of viable cells reduce a pale yellow MTT to a navy blue formazan, so if more viable cells are present in the well, more MTT will be reduced to formazan. When the incubation time was completed (4 h), 80 μ L of the lysing mixture was added to each well (lysing mixture: 225 mL dimethylformamide, 67.5 g sodium dodecylsulfate (both from Sigma) and 275 mL of distilled water). After 24 h, when formazan crystals had dissolved, the optical densities of the samples were read on an Elisa spectra max 190 photometer at 570 nm wavelength. Each compound at a given concentration was tested in triplicate in each experiment, which was repeated three times.

Inhibition of the carrageenin-induced edema [11]. Edema was induced in the right hind paw of Fisher 344 rats (150-200 g) by the intradermal injection of 0.1 mL 2% carrageenin in water. Both sexes were used. Pregnant females were excluded. Each group was composed of 6-15 animals. The animals, which have been bred in our laboratory, were housed under standard conditions and received a diet of commercial food pellets and water ad libitum during the maintenance but they were entirely fasted during the experiment period. Our studies were in accordance with recognised guidelines on animal experimentation.

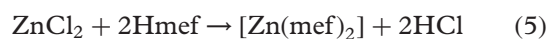
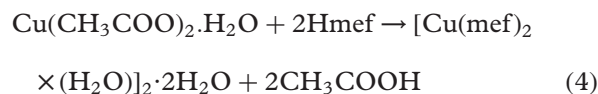
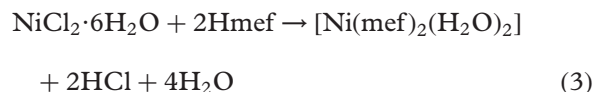
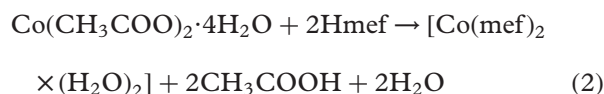
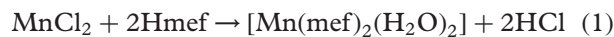
The tested compounds 0.01 or 0.1 mmol/kg body weight, were suspended in water, with few drops of Tween 80 and ground in a mortar before use or dissolved in water and were given intraperitoneally simultaneously. The rats were euthanized 3.5 h after carrageenin injection. The difference between the weight of the injected and uninjected paws was calculated for each animal. The change in paw weight was compared with that in control animals (treated with water) and expressed as a percent inhibition of the edema CPE % values Table V. Values CPE % are the mean from two different experiments with a standard error of the mean less than 10%.

Results and discussion

Synthetic aspects

We have prepared complexes of Mn(II), Co(II), Ni(II), Cu(II) and Zn(II) with mefenamic acid, 2-[(2,3-dimethylphenyl)amino]benzoic acid, HMef, in order to obtain information on structure-activity relationships for systems involving nickel(II), copper(II) and zinc(II) atoms. The complexes $[Mn(mef)_2 \cdot (H_2O)_2]$ (1), $[Co(mef)_2 \cdot (H_2O)_2]$ (2), $[Ni(mef)_2 \cdot (H_2O)_2]$ (3),

$[Cu(mef)_2 \cdot H_2O]_2 \cdot 2H_2O$ (4) and $[Zn(mef)_2]$ (5), were synthesized according to the reactions (1)–(5), in methanolic and aqueous solutions.



The complexes are microcrystalline or powder-like and stable in atmospheric conditions. All the complexes are insoluble in water, except of 1 which is slight soluble in water, in pentane and cyclohexane and all are soluble in the solvents Me_2CO , $CHCl_3$, CH_2Cl_2 , EtOH, DMSO, DMF, benzene and toluene. The elemental analyses confirm their stoichiometry. The μ_{eff} values of the complexes, show that these are all high spin; the large and small orbital contribution in the 2 and 3, $t_{2g}^5 e_g^2$ Co(II) and $t_{2g}^6 e_g^2$ Ni(II), respectively, indicate six-coordinate structures.

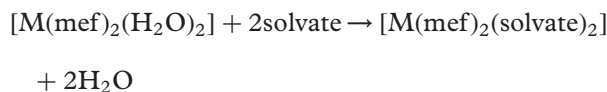
Spectroscopy

Infrared spectroscopy. As the carboxyl hydrogen is more acidic than the amino hydrogen the deprotonation occurs in the carboxylic group. This is confirmed by the IR spectra of the complexes, showing the characteristic bands for the secondary amino groups and for the coordinated carboxylato group. A broad absorption at 3500 cm^{-1} in the spectra of the complexes was attributed to the presence of coordinated water. The absence of large systematic shifts of the $\nu(NH)$ and $\delta(NH)$ bands in the spectra of the complexes compared with those of the ligand indicates that there is no interaction between the NH group and the metal ions. The $\nu_{as}(COO)$ and $\nu_{sym}(COO)$ bands of the prepared complexes are at 1615-1580 and at 1500-1450 cm^{-1} respectively; the difference, $\Delta[\nu_{as}(COO) - \nu_{sym}(COO)]$ between these frequencies for 1-3 and 5 is significantly less than the ionic value (for sodium mefenamic the Δ value is 180 cm^{-1}), as expected for the bidentate chelating mode of carboxylate ligation [7a,14]. The difference, $\Delta[\nu_{as}(COO) - \nu_{sym}(COO)]$ between these frequencies for 4 is close and less (161 cm^{-1}) to that for sodium mefenamic, as expected

for bridging bidentate carboxylato groups, [15] supporting a structure analogous to $[\text{Cu}(\text{mef})_2(\text{DMSO})_2]_2$, Figure 2 [10b] and $[\text{Cu}(\text{diclof})_2(\text{DMF})]_2$ [15]. The four carboxylate groups from four ligands are in a bidentate *syn, syn* $\eta^1:\eta^1:\mu^2$ bridging mode. The square pyramid geometry with a dimethyl formamide oxygen or water occupying both apical positions as was established by single crystal X-ray study [10b,15]. The compounds, 1-5 gave bands at $\sim 3300\text{ cm}^{-1}$ attributable to intramolecular hydrogen bonds $\text{NH}\dots\text{O}$. The medium bands at $\sim 400\text{ cm}^{-1}$ is attributed to the $\nu(\text{M}-\text{O}_{\text{H}_2\text{O}})$ stretching mode, while the bands at $250\text{--}210\text{ cm}^{-1}$ to the $\nu(\text{M}-\text{O}_{\text{oco}})$ stretching mode [14–16].

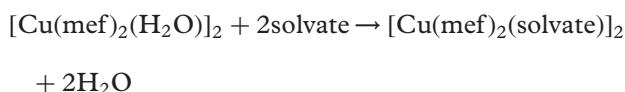
Electronic spectroscopy. The electronic spectra were recorded in DMF and CHCl_3 solution and absorption maxima in the uv-visible region are listed in Table I along with suggested assignments [14–16]. The electronic spectra of the Mn(II) complex, 1, can be assigned six coordinate stereochemistry [14]. The absorption of the organic ligand tailing into the visible region obscure the very weak d-d absorption bands of the manganese (II) complexes [14–17]. The d-d spectra of the solvated 1-3 can be assigned to transitions in pseudo-octahedral structures or six-coordinated tetragonally distorted stereochemistries. The band frequencies and the 10Dq value in 3 are characteristic of a Ni(II) O_6 chromophore, which do not cause a pronounced nephelauxetic effect [11,16]. The solution spectral data indicate the complexes 1-3 are all solvated with two solvent molecules

coordinating in a pseudo-octahedral arrangement.



Where M is Mn(II) or Ni(II) or Co(II) and solvate is DMF or CHCl_3 .

The solution spectra of 4 shows two bands having maxima at $\sim 14,000\text{--}14,900$ (band I) and at $22,500\text{--}25,000\text{ cm}^{-1}$ (band II), typical for square pyramidal species with CuO_5 chromophore. It is recognized that band I is assigned to a ligand field transition $d_{xz}d_{yz} \rightarrow d_{x^2-y^2}$ and band II to a charge transfer from carboxylato-oxygen atoms to the metal ion, without ruling out a transition of the type $d_{z^2} \rightarrow d_{x^2-y^2}$. The same pattern of spectrum is shown when compound 4 is dissolved in CHCl_3 and DMF, suggesting that these axial ligands exchanged with solvent molecules according to the reaction [14].



Biological studies

In vitro biological studies. In this investigation all compounds were studied in order to gain insight into their biological response. The metal complexes and the parent drug were studied with regard to their antioxidant ability as well as to the inhibition

Table I. Spectral data (UV-vis) and μ_{eff} values for the prepared complexes and the parent drug.

Compound	μ_{eff}	Solution (CHCl_3)			Solution (DMF)			Assignments
		$\lambda(\text{nm})$	$\nu(\text{cm}^{-1})$	$\log \epsilon$	$\lambda(\text{nm})$	$\nu(\text{cm}^{-1})$	$\log \epsilon$	
Mefenamic acid		356	28,090	3,72	351	28,490	3,77	$n \rightarrow \pi^*$
		282	35,461	3,76	284	35,211	3,92	$\pi \rightarrow \pi^*$
$[\text{Mn}(\text{mef})_2(\text{H}_2\text{O})_2]$	5.39	503sh	19,881	2,11	500sh	20,000	1,70	${}^6\text{A}_{1g} \rightarrow {}^4\text{T}_{1g}(\text{G})$
		430	23,255	2,45	370	27,027	3,34	${}^6\text{A}_{1g} \rightarrow {}^4\text{T}_{2g}(\text{G})$
		354	28,249	4,04	339	29,500	3,98	${}^6\text{A}_{1g} \rightarrow {}^4\text{T}_{2g}(\text{D})$
		283	35,336	4,13	292	34,246	4,27	$\pi \rightarrow \pi^*$
$[\text{Co}(\text{mef})_2(\text{H}_2\text{O})_2]$	4.53	574	17,422	1,65	568	17,606	1,83	${}^4\text{T}_{1g} \rightarrow {}^4\text{A}_{2g}$
		536	18,657	1,5	536	18,657	1,80	${}^4\text{T}_{1g} \rightarrow {}^4\text{T}_{1g}(\text{P})$
		355	28,169	3,97	340	29,412	4,00	$n \rightarrow \pi^*$
		283	35,336	4,03	291	34,364	4,28	$\pi \rightarrow \pi^*$
$[\text{Ni}(\text{mef})_2(\text{H}_2\text{O})_2]$	3.88	749sh	13,550	0,77	742sh	13,369	0,81	${}^3\text{A}_{2g} \rightarrow {}^3\text{T}_{1g}$
		680	14,706	0,88	680	14,706	0,85	${}^3\text{A}_{2g} \rightarrow {}^1\text{E}_g$
		378	26,455	2,36	400sh	25,000	2,99	${}^3\text{A}_{2g} \rightarrow {}^3\text{T}_{1g}(\text{P})$
		351	28,490	4,44	342	29,240	4,17	$n \rightarrow \pi^*$
		289	34,602	4,51	290	34,483	4,43	$\pi \rightarrow \pi^*$
$[\text{Cu}(\text{mef})_2(\text{H}_2\text{O})]_2$	2.02	672	14,880	2,24	715	13,986	2,34	d-d
		445	22,470	2,56	400	25,000	2,88	d-d
		355	28,169	3,93	334	29,940	3,98	$n \rightarrow \pi^*$
		282	35,461	4,03	291	34,364	4,03	$\pi \rightarrow \pi^*$

Table II. Interaction % with DPPH- Reducing ability (RA %).

Compounds	RA % 0.1 mM		RA % 0.5 mM	
	20 min	60 min	20 min	60 min
Mefenamic	2.1	2.6	7.5	8.3
[Mn(mef) ₂ (H ₂ O) ₂]	44.0	69.9	59.7	69.9
[Co(mef) ₂ (H ₂ O) ₂]	8.0	10.2	9.1	10.1
[Ni(mef) ₂ (H ₂ O) ₂]	6.2	8.1	13.0	13.1
[Cu(mef) ₂ (H ₂ O) ₂]	18.4	25.8	40.1	52.7
[Zn(mef) ₂]	3.8	8.5	5.2	18.8
MnCl ₂	4.2	4.1	3.2	3.5
CoCl ₂	3.1	2.7	2.7	2.9
NiCl ₂ ·6H ₂ O	3.1	6.1	5.1	4.9
CuCl ₂	4.6	5.6	4.9	5.8
ZnCl ₂	3.5	5.2	3.8	5.3
Ascorbic acid	99.5	99.9	94.5	93.9

of a) soybean lipoxygenase, b) β -glucuronidase and c) of trypsin- induced proteolysis.

Nowadays, antioxidants that exhibit DPPH radical scavenging activity is increasingly receiving attention. They have been reported to have interesting anticancer, anti-aging, and anti-inflammatory activities. Consequently, compounds with antioxidant properties could be expected to offer protection in rheumatoid arthritis and inflammation, and to lead to potentially effective drugs. In fact, many non-steroidal anti-inflammatory drugs have been reported to act either as inhibitors of free radical production or as radical scavengers. The model of the scavenging of the stable DPPH radical is extensively used to evaluate antioxidant activities in less time than other methods. DPPH is a stable free radical that can accept an electron or hydrogen radical and thus be converted into a stable, diamagnetic molecule. DPPH has an odd electron and so has a strong absorption band at 517 nm. When this electron becomes paired off, the absorption decreases stoichiometrically with respect

to the number of electrons taken up. Such a change in the absorbance produced in this reaction has been widely applied to test the capacity of numerous molecules to act as free radical scavengers. The reducing abilities of the complexes, 1-5 were evaluated using the 1,1-diphenyl-1-picrylhydrazyl, DPPH, free radical scavenging assay [11] in an iron-free system. This interaction expresses the reducing ability of the compounds. The scavenging activities of the complexes were measured and compared with those of the free drug and of vitamin C.

Not all the tested compounds were found to interact with the stable free radical DPPH, (Table II). The mefenamic acid and complexes 2, 3 and 5 present very low interactions (8,6.2 and 3.8%). It seems that interaction increases with the time and the concentration. Complex 4 possess mild interaction. Complex [Mn(mef)₂·(H₂O)₂] (1), presents the highest interaction with DPPH, which is time and concentration dependent.

During the inflammatory process, phagocytes generate the superoxide anion radical at the inflamed site, and this is connected to other oxidizing species such as HO·. Hydroxyl radicals are produced by reactions which are depended on transition metals, particularly iron. The competition of the compounds (0.1 mM) with DMSO for HO· generated by the Fe³⁺/ascorbic acid system, expressed as the inhibition of formaldehyde production, was used for the evaluation of their hydroxyl radical scavenging activity. In these experiments, (Table III), mefenamic acid exhibits the highest competition, compounds 2-5 show potent inhibition, while complex 1 is totally inactive. Trolox was used as a reference compound.

In this investigation all complexes were studied in order to gain insight their LOX-inhibition. Most of the recognised LOX inhibitors are antioxidants

Table III. Competition % with DMSO (0.1 mM) for hydroxyl radical HO·, inhibition of LOX and antiproteolytic activity of mefenamic acid and its metal complexes.

Compounds	Competition % with DMSO (0.1 mM)	Inhibition of LOX at 5 min (0.033 mM)	% Inhibition of Glucuronidase (0.1 mM)	% Inhibition of trypsin (0.3 mM)
Mefenamic acid	99.7	No	91.8	NA
[Mn(mef) ₂ (H ₂ O) ₂]	NA	94.7	NA	47.8
[Co(mef) ₂ (H ₂ O) ₂]	61	12.5	94.3	58.2
[Ni(mef) ₂ (H ₂ O) ₂]	67.7	39.4	78.3	34.4
[Cu(mef) ₂ (H ₂ O) ₂]	51.3	No	NA	31
[Zn(mef) ₂]	60.2	26.5	NA	23
MnCl ₂	NA	-	-	26
CoCl ₂	NA	-	-	27
NiCl ₂ ·6H ₂ O	NA	-	-	75
CuCl ₂	NA	-	-	24
ZnCl ₂	NA	-	-	-
Salicylic acid			56.4	IC ₅₀ 0.1 mM
trolox	88.2			

NA, no activity.

or free radical scavengers since lipoxygenation occurs via a carbon centered radical. The activity of the metal complexes and the “parent drug” to inhibit the enzyme soybean lipoxygenase has been studied. Mefenamic acid did not exhibit inhibition of the enzyme LOX. Perusal of the % inhibition values, that complex $[\text{Mn}(\text{mef})_2 \cdot (\text{H}_2\text{O})_2]$ (1), presents the higher activity, while complex 4 is totally inactive, (Table III).

The role played by proteases in the early stage of inflammatory process is well documented. Some anti-inflammatory agents have been reported to exhibit antiproteolytic activity [11]. The antiproteolytic activity of the metal complexes and the parent drug was studied. Complexes $[\text{Co}(\text{mef})_2(\text{H}_2\text{O})_2]$ (2) and $[\text{Ni}(\text{mef})_2(\text{H}_2\text{O})_2]$ (3) were found to inhibit significantly high β -glucuronidase, while complexes 1, 4 and 5 did not present any effect against this enzyme under the reported experimental conditions. Complexes 1 (47.8%) and 2 (58.2%), were found to inhibit trypsin *in vitro*. Mefenamic acid does not present any effect and complexes 3-5 (23-34%) were also found to be inactive under the reported experimental conditions, (Table III).

Antiproliferative activity in vitro [18]. The results of cytotoxic activity *in vitro* are expressed as IC_{50} – the concentration of compound (in μM) that inhibits a proliferation rate of the tumor cells by 50% as compared to control untreated cells, (Table IV). Mefenamic acid and the metal complexes 1-5 were tested for their antiproliferative activity *in vitro* against the cells of three human cancer cell lines: MCF-7 (human breast cancer cell line), T24 (bladder cancer cell line), A-549 (non-small cell lung carcinoma) and a mouse fibroblast L-929 cell line and the results are compared with the known chemotherapeutic *cis-platin*.

The IC_{50} value for for the complex $[\text{Cu}(\text{mef})_2(\text{H}_2\text{O})_2]$ against the T24 cell line is 7.77×10^{-6} M. The IC_{50} values shown by $[\text{Cu}(\text{mef})_2(\text{H}_2\text{O})_2]$ against MCF-7 and L-929 cancer cell lines are in a μM range similar to *cis-platin* 2.8 times and 8.0 times less cytotoxic against L-929 and MCF-7 cancer cell lines respectively.

These results indicate that coupling of **mefenamic acid** to Cu(II) metal center result in a metallic complex with important biological properties. The cytotoxic activity shown by $[\text{Cu}(\text{mef})_2(\text{H}_2\text{O})_2]$ against T24, MCF-7 and L-929 cancer cell lines indicates that coupling of **mefenamic acid** to Cu(II) metal center result in metallic complexes with important biological properties since it displays IC_{50} values in a M range similar or better to that of the antitumor drug *cis-platin*.

In vivo biological studies. The *in vivo* anti-inflammatory effects of the tested metal complexes and of their parent drug were assessed by using the functional model of carrageenin-induced rat paw edema and are presented in Table V as percentage of weight increase at the right hind paw in comparison to the uninjected left hind paw. Carrageenin- induced edema is a non-specific inflammation resulting from a complex of diverse mediators [19]. Since oedemas of this type are highly sensitive to non-steroidal anti-inflammatory drugs (NSAIDs), carrageenin has been accepted as a useful agent for studying new anti-inflammatory drugs [20]. This model reliably predicts the anti-inflammatory efficacy of the NSAIDs and during the second phase it detects compounds that are anti-inflammatory agents as a result of inhibition of prostaglandin amplification [21]. In Table V, is shown the protection observed against carrageenin –induced paw edema, by the investigated complexes (0.01 mmol/kg). The reference drug, mefenamic acid induced 61.5% protection at an equivalent concentration. The low solubility of $[\text{Cu}(\text{mef})_2 \cdot \text{H}_2\text{O}]_2$ prevents the measurement of CPE activity.

Actually the $[\text{Ni}(\text{mef})_2 \cdot (\text{H}_2\text{O})_2]$ was the most potent presenting toxic effects at 0.1 mmol/kg body weight. Thus, this compound was tested in lower concentration (0.01 mmol/kg). The inhibition caused by the complex $[\text{Ni}(\text{mef})_2 \cdot (\text{H}_2\text{O})_2]$ was found to be $17.5 \pm 0.4\%$ at 0.01 mmol/kg body weight BW. The complex $[\text{Zn}(\text{mef})_2]$ exhibited a strong inhibitory effect at 0.1 mmol/kg B.W. ($81.5 \pm 1.3\%$ inhibition), superior of the inhibition induced by mefenamic acid at the same dose ($61.5 \pm 2.3\%$ inhibition). The observed differences in activity seem to depend on the nature of the metal.

Table IV. The antiproliferative activity *in vitro* of mefenamic acid its metal complexes (expressed as IC_{50} (M) against MCF-7, T-24, A-549 and L-929 cancer cell lines.

Compounds	MCF-7 IC_{50} (M) \pm SD	T-24 IC_{50} (M) \pm SD	A-549 IC_{50} (M) \pm SD	L-929 IC_{50} (M) \pm SD
Mefenamic acid	14.92* \pm 3.1	8.12* \pm 4.5	16.83* \pm 2.3	17.82* \pm 2.8
$[\text{Mn}(\text{mef})_2(\text{H}_2\text{O})_2]$	7.26* \pm 3.5	3.51* \pm 1.0	<17.56*	<17.56*
$[\text{Co}(\text{mef})_2(\text{H}_2\text{O})_2]$	9.36* \pm 3.0	2.70* \pm 1.7	11.59* \pm 2.5	<17.44*
$[\text{Ni}(\text{mef})_2(\text{H}_2\text{O})_2]$	5.93* \pm 2.3	2.40* \pm 1.9	8.66* \pm 3.5	6.34* \pm 2.2
$[\text{Cu}(\text{mef})_2(\text{H}_2\text{O})_2]$	2.51* \pm 3.3	7.77** \pm 2.2	<10.03*	1.95* \pm 3.0
$[\text{Zn}(\text{mef})_2]$	4.07* \pm 5.0	3.77* \pm 2.0	11.12* \pm 3.0	7.44* \pm 1.0
Cis Pt	8.00**	41.7**	1.53**	0.70**

* $\times 10^{-5}$; ** $\times 10^{-6}$. Each value represents the mean obtained from 3 measurements (\pm SD).

Table V. Inhibition % of induced carrageenin rat paw edema (CPE %) at 0.1 mmol/kg.

Compounds	% CPE \pm SD
Mefenamic acid	61.5** \pm 2.3
[Co(mef) ₂ (H ₂ O) ₂]	71** \pm 1.1
[Ni(mef) ₂ (H ₂ O) ₂] ^a	17.5* \pm 0.4
[Zn(mef) ₂]	81.5* \pm 1.3

The effect on edema is expressed as percent of weight increase of hind paw (and as percent of inhibition of edema) in comparison to controls. Each value represents the mean obtained from 6 to 8 animals in two independent experiments (\pm SD). In all cases, significant difference from control: * $p < 0.1$ ** $p < 0.01$ (Student's T test); ^a This compound has been tested at 0.01 mmol/kg.

Conclusions

The complexes of mefenamic acid [Mn(mef)₂(H₂O)₂], [Co(mef)₂(H₂O)₂], [Ni(mef)₂(H₂O)₂], [Cu(mef)₂(H₂O)₂] and [Zn(mef)₂] were synthesized and characterized. Spectral studies reveal monomeric six-coordinated species for Mn(II), Ni(II) and Co(II), dimeric five-coordinated for Cu(II) and monomeric four-coordinated for Zn(II). In DMF or CHCl₃ solution the coordination number is retained and the coordinated molecules of water are replaced by solvent molecules.

The metal complexes and the "parent drug" have been studied with regard to their antioxidant ability as well as their inhibition of LOX, of β -glucuronidase and of trypsin- induced proteolysis. The complex [Mn(mef)₂(H₂O)₂] exhibits the highest antioxidant activity and the highest inhibitory effect against the soybean lipogxygenase (LOX), properties that are not demonstrated by mefenamic acid. Their antiinflammatory effects on rat paw edema induced by Carrageenan was studied and compared with those of mefenamic acid. The complex [Zn(mef)₂] exhibited a strong *in vivo* inhibitory effect at 0.1 mm/Kg B.W. (81.5 \pm 1.3%), superior than the inhibition induced by mefenamic acid at the same molar dose (61.5 \pm 2.3%). These compounds may prove useful for treating a variety of inflammatory diseases and may lead to the development of new drugs. Mefenamic acid and [Cu(mef)₂(H₂O)₂] are considered as agents with potential antitumor activity, and can therefore be candidates for further stages of screening *in vitro* and/or *in vivo*.

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