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Original Article

TRANSITION METAL COMPLEXATION WITH NAPROXEN AND EVALUATION OF THEIR *IN VITRO* ANTIMICROBIAL, CYTOTOXIC AND ANTHELMINTIC PROPERTIES

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ABSTRACT

Objective: The specific objective of this research was to synthesize transition metal complexes and investigate *in vitro* antimicrobial, cytotoxic and anthelmintic effects of naproxen and its complexes.

Methods: Naproxen was complexed with copper, cobalt iron, silver and zinc and the characterization of these compounds were performed by proton NMR, FTIR, DSC, SEM, HPLC and elemental analysis. The antimicrobial activity was evaluated by disc diffusion method and cytotoxicity was evaluated by using brine shrimp lethality bioassay and compared with vincristine sulfate. The anthelmintic activity was evaluated against *Pheritima posthuma*.

Results: Naproxen silver and zinc complexes showed potent activity while copper and cobalt complexes showed variable antimicrobial activity against some tested microorganisms. Naproxen iron complex showed moderate antimicrobial and very potent cytotoxic activity. According to these results we thought there might be any possibility to have anthelmintic properties on those complexes. However, we did not find any anthelmintic activity from them.

Conclusion: The present study demonstrated that few naproxen metal complexes possess comprehensive antimicrobial and cytotoxic activities.

Keywords: Naproxen, Antimicrobial, Cytotoxic, Anthelmintic.

INTRODUCTION

Coordination complexes of transition metals have been widely studied for their antibacterial, antifungal and potential cytotoxic chemotherapeutic agents. Metal complexes play an essential role in agriculture, pharmaceutical and industrial chemistry [1]. The use of metal complexes as therapeutic agents for treatment of different diseases has been extensively studied [2-6]. As they generally have the different mechanism of activity from the organic compounds to treat diseases, the development of metal complexes provides an alternative route of novel drug delivery system [7]. In addition to its ability to combat infection or neoplastic disease, these new agents must exhibit selective toxicity, chemical stability, and optimum rates of bio-transformation and elimination.

Coordination complexes of transition metal have a great historical perspective for their antimicrobial and antitumor (cytotoxic) properties. Many researchers have proved that the binding of a drug to metallo element enhances its activity and in many cases the complex possesses even such activity that the parent compound does not have any [8]. Thus we have motivated to study metal binding properties of naproxen derivatives with different transition metal ions and its antimicrobial, cytotoxic and anthelmintic activities.

To the best of our knowledge and available literature on the subject, no work has been done on the *in vitro* antimicrobial, cytotoxic and anthelmintic properties of naproxen metal complexes. In the present study, we report the synthesis of some organo metallic compounds of naproxen and evaluation of their few biological properties.

MATERIALS AND METHODS

Synthesis of sodium salt of naproxen (HL)

0.82 gm (0.1 M) of naproxen (Ligand) was dissolved with 0.1 M of sodium hydroxide solution in water to form the sodium salt of naproxen. Then the solution was sonicated for 5 min and kept in the room temperature. The potency of naproxen must be considered before preparation. The reaction mixture was put on a water bath to evaporate until a crystal film appeared; upon cooling the white product separated out.

General procedure for synthesis of transition metal complexes

Equimolar metal salts dissolved in water was added to the above mixture so that the ratio n(metal): n(ligand) of monovalent, divalent and trivalent ions used were 1: 1, 1: 2 and 1: 3 respectively in each case and immediate precipitation was occurred. Then the solid complexes were isolated by filtration, washed until free of chlorides with the corresponding solvent (methanol or water) and finally dried at room temperature.

The representative equations for the formation of the complexes can be presented as:

$$M^{n+}Cl_n \bullet hH_2O + nNaL = ML_n \bullet mH_2O + nNaCl + (h-m)\,H_2O$$

(Where, M = Co, Cu, Zn, Ag, Fe; n = 1 or 2 or 3; h= 0, 2, 4 or 6; m = 0 or 2 or 3).

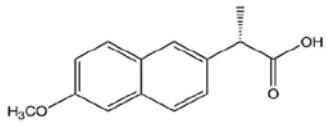


Fig. 1: Chemical structure of 6-methoxy--methyl–2-naphthalene acetic acid (Naproxen) (HL)

Chemicals and reagents

All chemicals were obtained commercially and were of analytical grade. Vincristine sulfate, used as a standard drug in cytotoxicity assay was collected from the Techno Drugs Limited, Bangladesh. Dimethyl sulfoxide (DMSO) purchased from Sigma-Aldrich, India. Sodium Chloride Crystal GR from Merck Ltd, Mumbai, India was used to prepare sea water in brine shrimp lethality bioassay. Albendazole was collected from SK+F Bangladesh Ltd. To the commercially available lyophilized Streptokinase vial from Beacon Pharmaceuticals Ltd, Bangladesh.

Antimicrobial activity

A total of 16 reference microbial strains (five Gram-positive, eight Gram-negative and three fungi) were used as the test organism for the antimicrobial screening. The antimicrobial activity of fractions against the test organisms was performed by disc diffusion method using standard disc (5 µg/disc) for comparison [9]. Ciprofloxacin was used as the standard disc for comparing antibacterial and

miconazole for antifungal activity. The test organisms were inoculated on 10 mL previously sterilized nutrient agar media, mixed thoroughly and transferred immediately to the sterile petri dish under an aseptic condition using a sterile loop. The paper discs containing the samples and standard disc were placed to the corresponding petri dish and were incubated for overnight at 37 °C. Clear zone of inhibition around the discs represented the presence of antimicrobial activity which was measured in millimeter (mm).

Collection of microorganisms

The microbial species used in the present study were *Bacillus cereus*, *Bacillus subtilis*, *Sarcina lutea*, *Staphylococcus aureus*, *Bacillus megaterium*, *Escherichia coli*, *Salmonella paratyphi*, *Salmonella typhi*, *Shigella boydii*, *Shigella dysenteriae*, *Pseudomonas aeruginosa*, *Vibrio mimicus*, *Vibrio parahemolyticus*, *Candida albicans, Aspergillus niger* and *Sacharomyces cerevacae.* These were collected as pure cultures from the Institute of Nutrition and Food Sciences, Dhaka University, Dhaka, Bangladesh.

Determination of cytotoxicity

The cytotoxic potentiality of all naproxen metal chelates was performed on brine shrimp nauplii using Mayer's method [10, 11]. The eggs of brine shrimp (*Artemia salina* Leach) were collected from the Department of Clinical Pharmacy and Pharmacology, University of Dhaka and hatched in a tank containing 1 L of simulated seawater at a temperature around 37 °C and pH 8.4 with constant oxygen supply. Two days were allowed to hatch and mature the nauplii. About 4 mg of each sample was dissolved in DMSO and solutions with varying concentrations (400, 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.563, 0.781 µg/ml) obtained by serial dilution technique. The prepared test solutions were added to the pre-marked vials containing 10 live brine shrimp nauplii in 5 mL simulated seawater and incubated for 24 h. After incubation period, the vials were examined using a magnifying glass in order to count the number of survived nauplii in each vial. From this data, lethality percent of the brine shrimp nauplii were calculated for each concentration. The median lethal concentration LC_{50} of each tested sample was calculated from the plotted graph of percentage of the shrimp mortality vs logarithm of the sample concentration, which was defined as the amount of sample required to kill 50% of brine shrimps within 24 h of exposure respectively.

Anthelmintic activity

The anthelmintic assay was carried out as reported earlier [12] with minor modifications. The naproxen metal chelates were dissolved in the minimum amount of DMF and the volume was adjusted to 10 ml with normal saline. All drugs and metal chelates solutions were freshly prepared before starting the experiment. 10 ml formulations containing three different concentrations (5, 10 and 20 mg/ml in normal saline) were prepared and six worms were placed in it. Observations were made for the time taken for paralysis (paralysis was said to occur when no movement of any sort could be observed except when worm were shaken vigorously) and death (time for death of worms was recorded after ascertaining that worms neither moved when shaken vigorously nor when dipped in warm water (50°C), followed with their body colors fading away). Albendazole (10 mg/ml) was used as reference standard while normal saline as the control.

Selection of worm

The assay was performed on adult earthworm, *Pheretima posthuma* due to its anatomical and physiological resemblance to the human intestinal round worm parasite [13, 14]. Because of easy availability, earthworms have been widely used for the initial evaluation of anthelmintic compounds *in vitro*. Adult earthworms (*Pheretima posthuma*) were collected from moist soil of Savar area of Dhaka, Bangladesh and washed with normal saline to remove all fecal matters. Then worms were used for anthelmintic study. The earthworms of 3-5 cm in lengths and 0.1-0.2 cm in width were used for all the experimental protocol.

Statistical analysis

All the values are expressed as mean ± SEM One way ANOVA followed by Dunnett's test was used to determine a significant difference between control groups and experimental groups. $P \leq 0.05$ were considered to be statistically significant. Regression analysis was carried out for analyzing the data obtained from brine shrimp lethality bioassay.

RESULTS

Physical, Analytical and thermal properties

All the complexes synthesized were crystalline solids and soluble in solvents but insoluble in ethanol and acetone. They were characterized by elemental analyses, IR spectra, thermal analysis, electronic photography (SEM), HPLC and magnetic properties (NMR). Table 1 shows the results of elemental and thermal analysis of the complexes. The melting points or decomposition temperatures of the chelates were found to be higher, which suggested their thermal stability. Naproxen decomposed at 153 °C where the complexes decomposed in the range of 218—250 °C (fig. 2a) followed by complete burning at above 700 °C.

Table 1: Physical, Analytical and thermal properties of naproxen metal complexes

Compound	Formula	Formula Weight	Color	Yield % (g)	Analysis (%) Calculated (Found)			Decomposition point (°C)
					C	H	M	
$(C_{14}H_{13}O_3)_2Cu.2H_2O$	$C_{28}H_{30}O_8Cu$	558.07	Green	84.7	60.21 (60.08)	5.38 (5.32)	11.39 (11.42)	227.51
$(C_{14}H_{13}O_3)_2Co.2H_2O$	$C_{28}H_{30}O_8Co$	553.45	Light red	75.8	60.71 (60.39)	5.42 (5.45)	10.65 (10.67)	242.62
$(C_{14}H_{13}O_3)_3Fe.3H_2O$	$C_{42}H_{45}O_{12}Fe$	797.63	Yellow	86.3	63.19 (63.12)	5.64 (5.63)	7.00 (6.97)	235.13
$(C_{14}H_{13}O_3)Ag.H_2O$	$C_{14}H_{15}O_4Ag$	355.13	White	85.3	47.30 (47.22)	4.26 (4.22)	30.38 (30.41)	218.89
$(C_{14}H_{13}O_3)_2Zn.2H_2O$	$C_{28}H_{30}O_8Zn$	559.9	White	78.3	60.01 (59.78)	5.36 (5.32)	11.68 (11.73)	225.28

NMR study

In case of 1H NMR spectrum of Naproxen, the protons of methyl (CH3) group showed a sharp doublet at ~δ 1.6, the methenyl (-CH) proton showed a triplet around δ 3.60-3.82, the methoxy (CH3O) protons, they exhibited a sharp singlet at δ 3.92 and the naphthyl protons appeared at δ 7.10-7.76 as a multiplet. All these protons shifted upfield in the complexes; the methenyl proton displayed the highest chemical shift δ 0.20-0.30 whereas the methoxy protons shift the least ~δ 0.05. This is due to the lesser electron withdrawing capacity of metal ions in the complexes relative to that of the carboxy proton in the ligand. The hydrogen atom of the–COOH group is absent in the metal complexes of 1H-NMR spectra (range of 10-13 ppm). This data indicated coordination and the carboxyl group is not protonated.

FTIR study

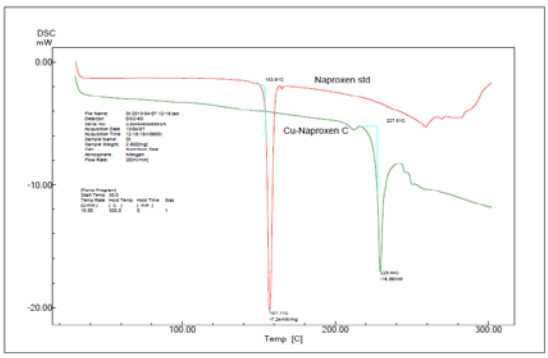
In the IR spectrum of Naproxen the ν(C=O) stretching mode of the carboxylic acid group was observed as a band at ν= 1729 cm^{-1}. When deprotonated it was disappeared and there were two new bands at 1545 and 1409, the carboxylate antisymmetric and symmetric

vibrations respectively (fig. 2(b) and 2 (c)). The carboxylate ion usually coordinates to metal ions in three main ways [15]. The values of Δ ν= νas(COO)-νs(COO) in monodentate complexes were expected to be much larger than 350 cm-1. When 200<Δν<350 cm-1 contain the carboxylate groups were present as an intermediate state between monodentate and bidentate, which was called anisobiden tate and when Δ ν<200 cm-1, the carboxylate groups of the complexes can be considered bidentate [15]. Such differences were reflected in the relative position of the antisymmetric and symmetric stretching vibrations. The main IR bands in the spectra of the sodium salt and the complexes were listed in table 2. A strong band was observed in the region 3190-3450 was certainly due to the absorption of crystal or coordination water. Assignment of the type of carboxylate group coordination was based on both the position of vas and vs bands and the values of Δν [16-20]. The values of all complexes lied in the range of 132-159 cm^{-1} which was close to that of naproxen sodium indicating that the carboxylate group functions as a bridging ligand. In solid state,the synthesized complexes of carboxylate mostly formed bridged dimers (M_2L_2 or M_2L_4) and also poly-meric networks [21].

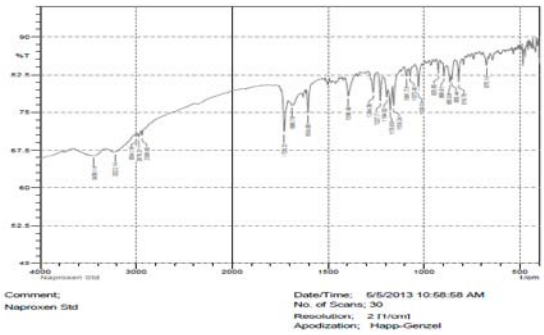
Table 2: Infrared data (cm-1) of Naproxen metal complexes in KBr

Compound	$\nu_{as}(COO^-)$			$\nu(H_2O)$
	ν_{as}	ν_s	$\Delta\nu$	
NaL	1545	1409	136	---
$CuL_2.2H_2O$	1554	1405	149	3406
$CoL_2.2H_2O$	1562	1415	147	3418
$FeL_3.3H_2O$	1601	1449	142	3422
$AgL.H_2O$	1610	1451	159	3388
$ZnL_2.2H_2O$	1544	1412	132	3386

Here, L= $C_{14}H_{13}O_3\neg$.



(a)



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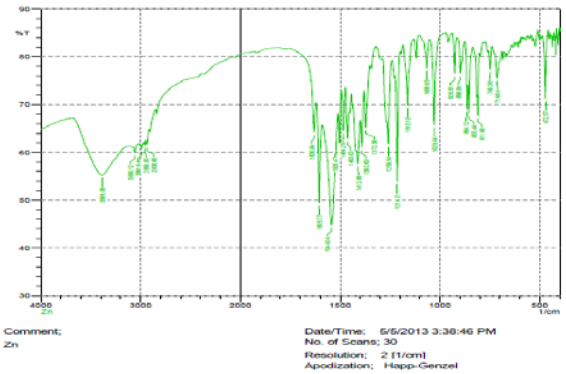
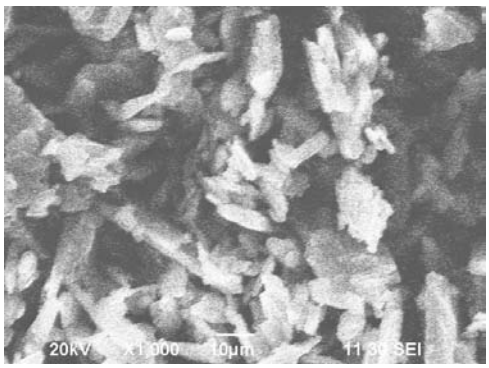


(c)

Fig. 2: (a) DSC thermogram of Naproxen and its copper complex, (b) and (c) IR spectrum of Naproxen and its copper complex respectively

Scanning electron microscopy

Scanning electron microscope (SEM) images were taken in order to study the surface morphology of Naproxen metal complexes. The SEM micrographs of ligand and its complexes are shown in (fig. 3). The images showed particles with fiber-like morphology of the complexes compared to ligand (Naproxen) which was homogenously distributed solid powder. The photograph clearly indicated that the complexes were hydrated and they formed dimer or even polymeric networks in the micrometer range.



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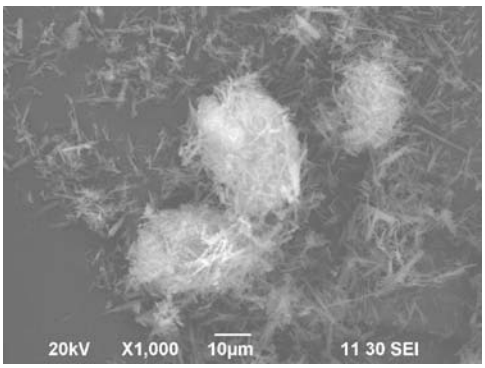

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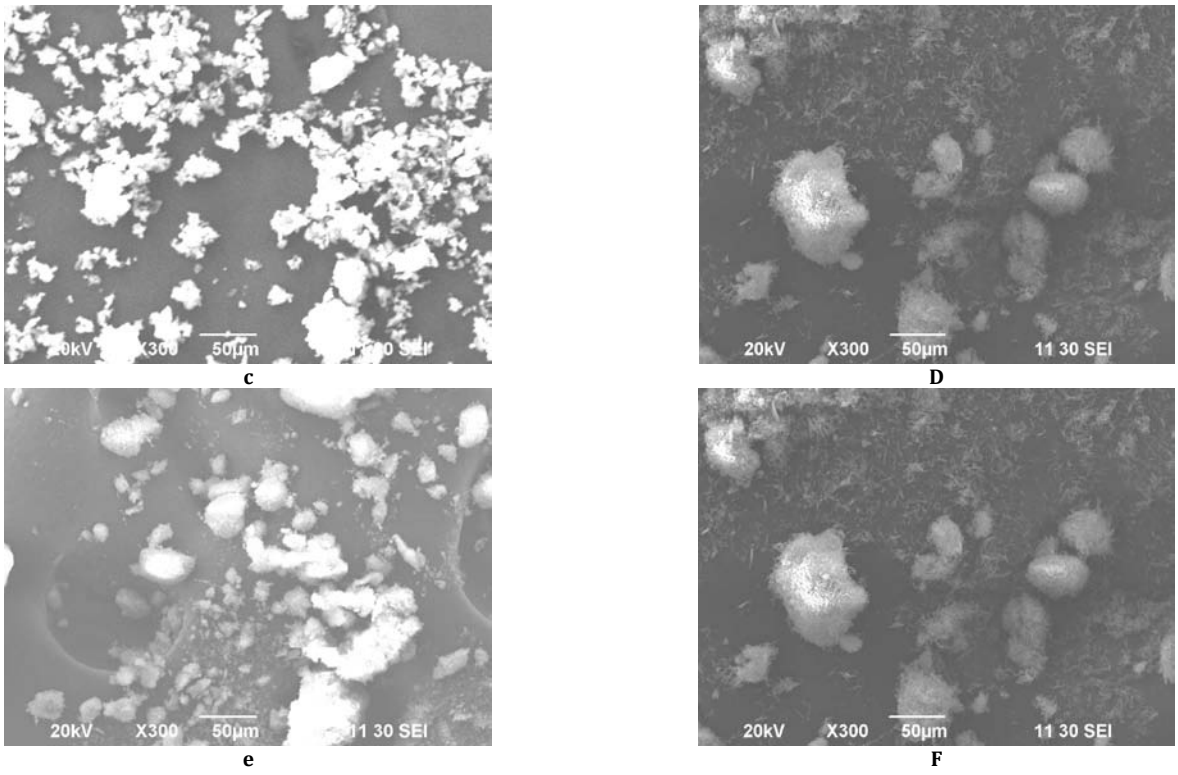

Fig. 3: Scanning electron microscopy (SEM) photomicrograph of (a) Naproxen, (b) Naproxen Copper complex (N-Cu), (c) Naproxen Cobalt complex (N-Co), (d) Naproxen Iron complex (N-Fe), (e) Naproxen Silver complex (N-Ag) and (f) Naproxen Zinc complex (N-Zn) respectively

HPLC study

The liquid RP-chromatography studies were performed in order to determine purity of the new synthetic products in comparison to the free ligand (Naproxen). Acetonitrile and water in various ratios were used as mobile phases.

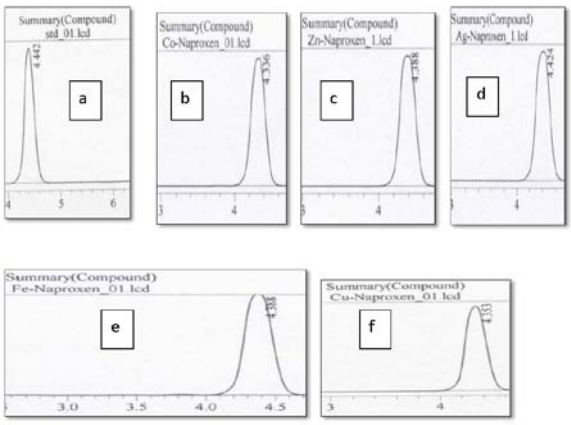

Fig. 4: HPLC analysis of (a) Naproxen, (b) Naproxen Cobalt complex (N-Co), (c) Naproxen Zinc complex (N-Zn), (d) Naproxen Silver complex (N-Ag), (e) Naproxen Iron complex (N-Fe), and (f) Naproxen Copper complex (N-Cu) respectively

Analytical method

High Performance Liquid Chromatographic system (Shimadzu-UFLC Prominence), equipped with an auto sampler (Model-SIL 20AC HT) and UV-Visible detector (Model-SPD 20A) was used for the analysis of the samples. The data were recorded using LC-solutions software. Analytical reversed phase C-18 column [4.6 x 150 mm, 5 µm] was used to analyze the standards and samples. The HPLC assay method described in United States Pharmacopoea (USP35 NF30, volume-3, Page-3996, 2012) was used to analyze the samples.

Chromatograms of the ligands and products

HPLC methods were used to confirm the appearance of new products after the synthesis had been performed. The samples of ligand and complexes eluted close to each other with similar retention times. The complexes were purified with the standard analytical Nucleosil column. The samples of the ligand and complexes were injected into the 20 µl loop and were sufficient to obtain a clean chromatogram and the identification of the complexes was satisfactory. The chromatographic data for the complexes and their ligands are given in the table 3.

Table 3: Retention times of the complexes and their ligand

Compound	Retention time(t_r)(min)
Naproxen(ligand)	4.442
Naproxen Copper complex (N-Cu)	4.353
Naproxen Cobalt complex (N-Co)	4.336
Naproxen Iron complex (N-Fe)	4.388
Naproxen Silver complex (N-Ag)	4.424
Naproxen Zinc complex (N-Zn)	4.388

The studies indicated that HPLC is also a good tool for identification of the metal complexes. It is extremely useful when no X-ray data of the compounds are available.

Antimicrobial activity

The results of different metal chealates of naproxen with disc diffusion method are shown in (table 4). The antimicrobial activity of all test fractions was tested using concentration 400 µg/disc. Naproxen cobalt and silver complexes showed considerable antimicrobial activity on various microorganisms. A naproxen copper and zinc complex has moderate activity and iron complex does not show any comparable antimicrobial activity.

Table 4: Antimicrobial activity of naproxen and metal complexes

Test microorganisms	Zone of inhibition (in mm by transparent scale)						
	Ciprofloxacin	Naproxen	N-Cu	N-Co	N-Fe	N-Ag	N-Zn
Gram positive bacteria							
Bacillus cereus	32	-	14	13	5	18	21
Bacillus megaterium	31	-	12	15	3	16	18
Bacillus subtilis	30	-	17	15	7	21	17
Staphylococcus aureus	28	-	6	10	8	19	12
Sarcina lutea	31	-	-	-	9	17	15
Fungi (Miconazole as standard)							
Candida albicans	28	-	10	9	-	15	13
Aspergillus niger	24	-	7	-	-	13	16
Aspergillus flavus	25	-	9	-	8	16	18
Sacharomyces cerevacae	22	-	12	7	6	19	10

Table 4: Antimicrobial activity of naproxen and metal complexes (Continued)

Test microorganisms	Zone of inhibition (in mm by transparent scale)						
	Ciprofloxacin	Naproxen	N-Cu	N-Co	N-Fe	N-Ag	N-Zn
Gram negative bacteria							
Escherichia coli	37	-	11	17	24	20	8
Pseudomonas aeruginosa	33	-	9	14	13	17	7
Salmonella paratyphi	28	-	-	15	16	14	10
Salmonella typhi	26	-	-	-	12	9	4
Shigella boydii	29	-	13	-	-	11	6
Shigella dysenteriae	27	-	14	-	-	13	9
Vibrio mimicus	30	-	3	5	6	7	3

Cytotoxic activity

In brine shrimp lethality bioassay, percentage of mortality increased gradually with the increase in concentration of the test samples. The lethal concentration (LC_{50}) of the test samples after 24 h was obtained by a plot of percentage of the shrimps died against the logarithm of the sample concentration (toxicant concentration) and the best-fit line was obtained from the curve data by means of regression analysis. Vincristine Sulphate (VS) was used as positive control and the LC_{50} for VS was found as 0.544µg/ml. Compared with the negative control, VS (positive control) gave significant mortality.

In Brine Shrimp Lethality bioassay, varying degree of lethality to naproxen metal chelates was observed with exposure to different dose levels of the test samples (table 5 and 6). Copper, cobalt, silver and zinc complexes showed reduced cytotoxicity compared to parent naproxen whereas naproxen iron complex with the LC_{50} value of 0.205 µg/ml displayed greater activity compared to the Vincristine Sulphate (LC_{50} value 0.544 µg/ml). Naproxen iron complex showed very potent cytotoxic activity than standard and thus further other ways of cytotoxicity study would be performed in future.

Table 5: Percent of mortality of the nauplii of naproxen metal complexes

S. No.	Concentration (C)µg/ml	Mortality (%)					
		Naproxen	N-Cu	N-Co	N-Fe	N-Ag	N-Zn
1	400	100	20	20	100	20	20
2	200	100	10	20	100	10	10
3	100	100	10	10	100	10	10
4	50	100	0	0	100	10	0
5	25	100	0	0	100	0	0
6	12.5	60	0	0	100	0	0
7	6.25	50	0	0	100	0	0
8	3.125	20	0	0	70	0	0
9	1.5625	0	0	0	50	0	0
10	0.78125	0	0	0	50	0	0

Table 6: LC50 values of the test samples of naproxen metal chelates

Test sample	LC_{50} µg/ml
Vincristine Sulphate	0.544
Naproxen	8.96
N-Cu	14.25
N-Co	13.94
N-Fe	**0.205**
N-Ag	13.88
N-Zn	14.55

Anthelmintic activity

The effect of naproxen metal complexes at different conc.(mg/ml) to paralyze and cause death to earthworm to evaluate *in vitro* anthelmintic activity were observed as follows as shown in (table 7).

The anthelmintic activity of naproxen metal complexes was observed in a dose dependent manner. It is evident that naproxen and its metal complexes exhibited no anthelmintic activity in the dose-dependent manner giving very long time of paralysis and death with all concentrations. This revealed that the synthetic compound showed no anthelmintic activity in therapeutic medicine.

Table 7: In-vitro anthelmintic activity of Naproxen metal complexes

Group	Concentration	Time for paralysis in minutes	Time for death in minutes
Standard	5 mg/ml	128.17±1.57	192.50±3.06
	10 mg/ml	114.17±1.79	165.83±2.50
	20 mg/ml	68.17±2.30	104.00±2.73
Copper-Naproxen	5 mg/ml	147.83±1.35	212.33±2.48
Complex	10 mg/ml	138.00±0.81	191.33±1.85
	20 mg/ml	117.83±1.51	138.83±2.21
Cobalt-Naproxen	5 mg/ml	143.33±3.29	209.17±2.52
Complex	10 mg/ml	137.33±1.05	188.67±1.67
	20 mg/ml	114.33±1.39	141.50±1.70
Iron-Naproxen	5 mg/ml	160.83±2.55	219.67±2.16
Complex	10 mg/ml	142.67±1.35	195.67±3.23
	20 mg/ml	120.67±1.67	151.83±1.97
Silver-Naproxen	5 mg/ml	151.83±1.61	227.50±4.96
Complex	10 mg/ml	143.17±2.21	210.50±4.13
	20 mg/ml	125.83±3.87	163.83±3.99
Zinc-Naproxen	5 mg/ml	145.00±1.83	202.83±2.16
Complex	10 mg/ml	130.67±1.88	189.17±2.90
	20 mg/ml	111.33±2.70	145.67±1.56
Naproxen	5 mg/ml	153.83±1.61	201.50±1.86
	10 mg/ml	142.50±1.61	179.83±2.10
	20 mg/ml	125.00±2.97	152.50±2.81

DISCUSSION

Naproxen silver complex showed potent antimicrobial activity against most of the tested microorganisms compared to other complexes. Naproxen zinc complex showed better activity against gram positive strains than gram negative. Iron complex showed potent antimicrobial activity against gram negative *E. coli* than other strains. Copper and cobalt complexes had variable moderate activity against few microorganisms. According to Hard–soft acid base theory (HSAB theory) soft acids like Ag(I) and borderline acids (such as Co(II), Cu(II) and Zn(II)) tend to associate tightly with soft bases, such as the sulphydryl (R‑SH) groups that are found in proteins. Consequently, the anti bacterial toxicity of these metals is approximately proportional to their affinity for S atom [22–24].

Naproxen iron complex showed surprisingly very potent cytotoxic activity compared to vincristine sulphate while other metal complexes displayed reduced cytotoxicity than parent naproxen. Antimicrobial and cytotoxic activity of iron obtained here demonstrated that iron had inhibitory effects on bacterial and cellular growth. These findings are in agreement with results obtained from another studies of electron microscopy suggested that the integrity of the cytoplasmic membrane was severely compromised by exposure to toxic doses of iron [25, 26]. Based on these results, we predicted that there might be any anthelmintic activity of these compounds however, there was nothing at all.

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CONFLICT OF INTERESTS

We declare that we have no conflict of interest

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