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***In vitro* Antimicrobial and Cytotoxic Activities of Ferrocene Derivative Compounds**

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Abstract: The aim of the present study was to investigate the biocidal activity of four new ferrocene derivative based coordination complexes, [1,2,3-trithia-3]-ferrocenophane, C₁]; [disodium-1,1'-ferrocenedithiolate, C₂]; [1,1'-(ferrocene-1,1'-diyl)-diethanol, C₃]; [ferrocenyl(ethyl-phenyl)-methanol, C₄]; against microbes (bacteria and fungi) and brine shrimp (*Artemia salina* Lech.) nauplii. C₂ showed modest antibacterial activity at the concentration of 200 µg disc⁻¹ and gave its Minimum Inhibitory Concentration values at 32-64 µg mL⁻¹ against the tested bacteria. These complexes gave comparatively better antibacterial activity against the Gram-positives than the Gram-negatives. C₃, C₁ and C₄ showed moderate antifungal activity at concentration of 200 µg disc⁻¹. Brine shrimp eggs were hatched in artificial sea water and exposed to the complexes. Median lethal concentration (LC₅₀) values were calculated. Both complexes showed toxicity against brine shrimp but complex C₃ explored its potent cytotoxicity having LC₅₀ values at 6.031 µg mL⁻¹ (ppm).

Key words: Ferrocene derivatives, antimicrobial activity, *Artemia salina* L.

INTRODUCTION

The frequency of life threatening infections caused by pathogenic microorganisms is increased worldwide and is becoming an important cause of morbidity and mortality in immunocompromised patients in the developing countries (Black *et al.*, 1982, Walsh *et al.*, 1974). Although huge numbers of antibiotics have been discovered, these antibiotics are developing resistance to the pathogenic organism day by day. In third world countries like Bangladesh, irrational use of antibiotics is a major cause of such resistance. So it is no doubt important to discover newer, safer and more effective antibiotics. Biological and medicinal properties of transition metal complexes and their mechanisms of action is now a valuable tool for the modern drug discovery program. This topic has been dominated in recent years by the use of iron complexes in the clinical trial of cancer but covers a broad field ranging from effects on viruses, bacteria, use of gold complexes in arthritis and even nitroprusside as a vasodilator. Platinum based complex Cisplatin is one of the most effective drugs for treating testicular, ovarian, bladder and neck cancers. Despite the success of cisplatin, however, it lacks selectivity for tumour tissue, which leads to severe side effects. These include renal impairment, neurotoxicity and ototoxicity.

Now various tumour cell lines are growing resistance to cisplatin e.g., acquired cisplatin resistance in some (mainly murine) preclinical tumour models (Kelland, 1993). These problems have led the scientists to explore new and potent bioactive complexes which may come in the modern clinical trial. Synthetic chemical compounds constitute important sources of various bioactive compounds such as antimicrobial (Kamalakkannan and Venkappayya, 2002; Biswas *et al.*, 2000; Sultana *et al.*, 2003) and anticancer compounds (Amirkhanov *et al.*, 1999; Brown *et al.*, 1982; Rho *et al.*, 2002; Kelland *et al.*, 1994). Every year thousands of compounds are synthesized and many of them are subjected to pharmacological screening to determine if they have useful biological activity. Ferrocene derivative compounds have also been reported for their potent antimicrobial activity (Domarle *et al.*, 1998; Pradines *et al.*, 2001). As a part of our continuing research to obtain potential therapeutic agents we have synthesized four new ferrocene derivative based coordination complexes, [1, 2, 3-trithia-3]-ferrocenophane]; [disodium-1, 1'-ferrocenedithiolate]; [1, 1'-(ferrocene-1, 1'-diyl)-diethanol]; [ferrocenyl (ethyl-phenyl)-methanol,] So, the aim of the present study was to investigate the cytotoxicity as well as antimicrobial properties of four new ferrocene complexes with the hope of adding new and

potent chemotherapeutic agents to arsenal of weapons used against resistance organisms as well as against other most lethal infectious diseases like cancer, AIDS, SARS etc.

MATERIALS AND METHODS

Sources of the mixed ligand complexes: The compounds used in the present study were synthesized according to the following procedures;

Preparation of [1, 2, 3-trithia-[3]-ferrocenophane, C₁]: About 11.1 mL (74 m moles) of TMEDA in anhydrous hexane was poured in a 3 L flask equipped with condenser containing an argon inlet tube and a magnetic stirrer. To this mixture, 45.25 mL (74 m moles) of n-butyllithium in hexane solution was added through a syringe and then 10 min later a solution of 5.60 g (60 m moles) of ferrocene in 250 mL of anhydrous hexane was mixed with rapid stirring over a period of 1 h and then slowly 16 h. The orange crystals of 1, 1'-dilitionniferrocene which had separated by filtering with filter paper, washed with anhydrous hexane until the washings were colorless and then dried under high vacuum.

1, 1'-dilitionniferrocene and TMEDA (9.5 g, 0.305 moles) were mixed in a 500 mL round-bottomed flask equipped with a side armed reflex condenser and a magnetic stirrer. The addition of dry 1, 2- dimethoxyethane (300 mL) gave a clear, deep orange red solution and then sublimed sulfur (6.0 g, 187 mmoles) was added to cause warm and darken it. The resulting mixture was refluxed for 12 h, cooled and then filtered through a bed of celite. The filtrate was treated with diethyl ether (150 mL) and washed with 10% aqueous sodium hydroxide (5×100). The combined aqueous fractions were washed with diethyl ether (3×200) and then solvent removed by vacuum distillation to give a dark semisolid. This compound was analyzed by Thin Layer Chromatography (TLC) and showed two major products and traces of three other compounds. By using column chromatography with solvent system hexane: dichloromethane = 4:1 to give [1, 2, 3-trithia-[3]-ferrocenophane, C₁], an orange crystalline mass yielding value about 48%. ¹H NMR (CDCl₃, MHz): δ = 4.40 (H²), 4.30 (H³), 4.47 (H⁴), 3.75 (H⁵); 2C₂H₄ ppm. IR (KBr): ν = 1404 w, 1386 w, 1381 w, 1194 w, 1160 s, 1026 s, 1018 w, 891 s, 847 s, 833 s, 817 s, 611 m, 525 s, 492 s, 424 s.

Preparation of [disodium-1, 1'-ferrocenedithiolate, C₂]: 1, 2, 3-trithia-[3]-ferrocenophane (3.55 g, 127 mmoles) was added in a suspension of LiAlH₄ (1 g, 26 m moles) in diethyl ether (200 mL). The reacting mixture became warm

and evolved hydrogen sulfide. After stirring for 2 h under reflux, the reacting mixture was poured onto 300 mL of ice-cold water. The addition of potassium hydroxide (5 g) gave an orange aqueous layer and a yellow ether layer. The aqueous layer was separated from diethyl ether layer and then acidified with concentrated HCl which caused the product to separate as a bright yellow suspension. The suspended material in aqueous layer was extracted with 300 mL of ether and then the solvent was removed in vacuum to give the product as bright yellow crystals of ferrocene-1, 1'-dithiol. To a suspension of ferrocene -1,1'-dithiol (0.34 g, 1.4 m moles) in methanol (50 mL) was added a 40% aqueous solution of sodium hydroxide (0.23 mL) under stirring. The solution became red of disodium-1, 1'-ferrocenedithiolate, C₂] yielding value 3.05g (95%). ¹H NMR (CDCl₃, MHz): δ = 4.18 (m), 4.29 (m) and 4.44 (m) (4H, 2H, 2H, 2C₂H₄); 5.3 (SH) ppm. IR (KBr): ν = 2519 s, 2341 w, 1653 m, 1558 s, 1414 s, 1383 m, 1167 s, 1019 s, 892 s, 814 s, 506 m triplet.

Preparation of [1, 1'-(ferrocene-1,1'-diyl)-diethanol, C₃]: A solution of 1, 1'-diacetylferrocene (3.00 g, 11.0 mmoles) in dry tetrahydrofuran (40 mL) was added with stirring to a cooled solution of LiAlH₄ (0.30 g) in dry tetrahydrofuran (50 mL). The mixture was refluxed for 2 h and then ethyl acetate was added to destroy excess reductant. The reaction mixture was added in water (1.2 mL), ethanol (8.3 mL) and diethyl ether (16.6 mL): the resulting suspension was filtered through Hyflo-supercel and the filtrate was dried over Na₂SO₄. After removal of the solvent, the resulting deep yellow oil was dissolved in hot hexane: slow cooling of this solution gave 1,1'-(ferrocene-1,1'-diyl)-diethanol (87% yield) as deep yellow plates, m.p. 69-70°C and was additionally recrystallised eight times from hexane as yellow needle m.p. 99-100°C. Found: C, 62.0; H, 6.7. C₁₄H₁₈FeO₂ requires: C, 61.3; H, 6.6%. δ_H 1.52 (d, J = 6.3 Hz, 6H, 2 × CH₃); 1.71 (s, br., 2H, 2 × OH); 3.81 (q, J = 6.3 Hz, 2H, 2 × CH); 4.02 (m, 2H), 4.4 (m, 2H) and 4.25 (m, 4H) (C₂H₄); δ_C 22.2 (q, CH₃); 65.1 (d, CH); 68.2 (d), 70.1 (d), 71.2 (d) and 90.5 (s) (C₂H₄); 72.1 (d), (CHOH).

Preparation of [ferrocenyl(ethyl-phenyl)methanol C₄]: Ethyllithium (1.32 mL of a solution in hexane, 5.30 mmoles) was added with stirring to a solution of benzoylferrocene (1.59 g, 5.50 m moles) in dry ether (50 mL). The mixture was stirred for 15 h at room temperature and then poured onto a mixture of crushed ice (100 g) and dilute sulphuric acid (2 mol/100 L); when the ice had melted the mixture was extracted with ether (2×50 mL). The combined ether extracts were washed with water and dried over Na₂SO₄ before removal of the solvent. Recrystallisation from CH₂Cl₂ gave dark orange

crystals of ferrocenyl (ethyl-phenyl) methanol, (yield 96%), m.p. 110-111°C (from CH₂Cl₂/light petroleum). Found C, 71.95; H, 6.2. C₁₉H₂₀FeO requires: C, 71.3; H 6.2%. δ_H 0.82 (t, J = 6.0 Hz, 3H, CH₃); 2.10 (q, J = 6.0 Hz, 2H, CH₂); 2.59 (s, 1H, OH); 4.00 (m, 1H), 4.10 (m, 1H); 4.16 (m, 1H) and 4.39 (m, 1H) (C₅H₄); 4.4 (s, 5H, C₅H₅); 7.2-7.6 (m, 5H, C₆H₅); δ_C 8.0 (q, CH₃); 35.8 (t, CH₂); 66.4 (d), 66.5 (d), 67.6 (d), 67.9 (d) and 101.2 (s) (C₅H₄); 68.2 (d, C₅H₅); 74.4 (s, C-OH); 125.4 (d), 126.0 (d), 127.5 (d) and 145.3 (s) (C₆H₅).

Antibacterial screening: *In vitro* antibacterial screening is generally performed by disc diffusion method (Bauer *et al.*, 1966; Rios *et al.*, 1988) for primary selection of the compounds as therapeutic agent. The method is essentially a qualitative or semi quantitative test indicating sensitivity or resistance of microorganisms to the test materials as well as bacteriostatic or bactericidal activity of a compound (Carson *et al.*, 1995). The antibacterial activity of the complexes C₁, C₂, C₃ and C₄ were determined at a concentration of 30 $\mu\text{g disc}^{-1}$ and 200 $\mu\text{g disc}^{-1}$ against three gram-positive (*Bacillus subtilis*, *Streptococcus β -haemolyticus* and *Staphylococcus aureus*) and five gram-negative (*Pseudomonas aeruginosa*, *Escherichia coli*, *Salmonella typhi* and *Shigella dysenteriae*, *Klebsiella* species) bacteria. The diameters of zone of inhibition produced by the complexes were compared with the standard antibiotic (Ciprofloxacin 30 $\mu\text{g disc}^{-1}$).

Minimum Inhibitory Concentration (MIC) determination: A current definition of the Minimum Inhibitory Concentration, MIC, is the lowest concentration which resulted in maintenance or reduction of inoculum viability (Carson *et al.*, 1995). The determination of the MIC involves a semi quantitative test procedure which gives an approximation to the least concentration of an antimicrobial needed to prevent microbial growth. The method displays tubes of growth broth containing a test level of preservative, into which an inoculum of microbes was added. The end result of the test was the minimum concentration of antimicrobial (test materials) which gave a clear solution, i.e., no visual growth (Collins, 1964; Davidson and Parish, 1989). Serial dilution technique (Reiner, 1982) was applied for the determination of minimum inhibitory concentration of the complexes against the four tested bacteria, *Bacillus subtilis*, *Streptococcus β -haemolyticus*, *E. coli*, *Salmonella typhi* and *Klebsiella* species. The media used in this respect were nutrient broth (DIFCO). Dilution series were setup with 2, 4, 8, 16, 32, 64, 128, 256, 512 and 1024 $\mu\text{g mL}^{-1}$ of nutrient broth medium. To each tube 100 μL of

standardized suspension of the testing bacteria (10^7 cell/ mL) were added and incubated at 30°C for 24 h.

Collection of the bacterial species: The bacterial species used in this experiment were *Bacillus subtilis*, *Streptococcus β -haemolyticus*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *E. coli*, *Salmonella typhi*, *Shigella dysenteriae* and *Klebsiella* species all of which were collected from the Institute of Nutrition and Food Sciences (INFS), Dhaka University, Bangladesh.

Antifungal screening: The antifungal activity of the complexes were tested against the three pathogenic fungi *Candida albicans*, *Aspergillus niger* and *Aspergillus flavus* at a concentration of 200 $\mu\text{g disc}^{-1}$ for each. The activity was assessed by disc diffusion method (Bauer *et al.*, 1966). The media used in this respect was Potato Dextrose Agar (PDA). The activity was determined after 72 h of incubation at room temperature (30°C).

Cytotoxicity bioassay: Brine shrimp lethality bioassay (Persoone, 1980; Mayer *et al.*, 1982; McLaughlin and Anderson, 1988; McLaughlin, 1990) was carried out to investigate the cytotoxicity of the novel gold based complexes. Here, *in vivo* lethality test were carried out using brine shrimp nauplii eggs (*Artemia salina* Lech.). Eggs were placed in one side of a small tank divided by a net containing 3.8 % NaCl solution for hatching. In other side of the tank, a light source was placed in order to attract the nauplii. After two days of hatching period the nauplii were ready for the experiment. Then 3 mg of each complex was accurately measured and dissolved in 0.6 mL (600 μL) of DMSO to get a concentration of 5 mg mL⁻¹. From the stock solutions 0.5, 1, 2, 5, 10, 20 and 40 μL were placed in 7 different vials making the volume up to 5 mL by NaCl solution. The final concentration of the samples, in the vials became 0.5, 1, 2, 5, 10, 20 and 40 $\mu\text{g mL}^{-1}$ (ppm), respectively.

10 brine shrimp nauplii were then placed in each vial. For the control test of each vial, one vial containing the same volume of DMSO plus seawater up to 5 mL was used. After 24 h of incubation, the vials were observed using a magnifying glass and the number of survivors in each vial were counted and noted. The resulting data were transformed to the probit analysis (Finney, 1988) for the determination of LC₅₀ values for the extracts.

RESULTS AND DISCUSSION

Among the four ferrocene derivative complexes, compound C₃ showed substantial activity against both Gram-negative and Gram-positive bacteria at the concentration of 200 $\mu\text{g disc}^{-1}$ (Table 1). The compounds

did not show remarkable activity at the low concentration of $30 \mu\text{g disc}^{-1}$ compared with the standard ciprofloxacin. The compounds showed comparatively better activity against the Gram-negative bacteria than the Gram-positives. But the compound C_3 showed maximum zone of inhibition of 22 mm against the Gram-positive *Streptococcus* β -haemolyticus whereas the compound C_1 and C_4 gave maximum zone (18 mm) against the Gram-negative bacteria *Shigella dysenteriae* and *Klebsiella* species. However, another compound C_2 showed moderate antibacterial activity against both Gram-positive and Gram-negative bacteria. These findings indicate the susceptibility of ferrocene derivative complexes comparatively more against the Gram-positive bacteria. So the structural activity correlation (Fig. 1) of the obtained antibacterial screening data revealed that the compound C_3 was got highest activity and compound C_1 was the second highest among the tested compounds. Compound C_3 contain 1, 1' diyl diethanol and compound C_1 contains 1, 2, 3 trithiol group in cyclic form and compound C_2 contains two sodium conjugated thiol group in acyclic form. Therefore the position, cyclic form and the attachment of other groups are playing an important role to contribute antimicrobial activity. Since compound C_4 contains one ethyl, one phenyl and methanol groups but showed lowest antibacterial activity than the other compounds. From the present observation it is revealed that the diethanol group and cyclic form of thiol are contributing strong antibacterial activity of compounds C_3 and C_1 . Compound C_2 and C_4 have a moderate antimicrobial activity but less than that of other compounds.

The MIC values of the compound C_1 against *Bacillus subtilis*, *Streptococcus* β -haemolyticus, *Escherichia coli*, *Salmonella typhi* and *Klebsiella* species were found to be 64, 64, 64, 128 and 64 $\mu\text{g mL}^{-1}$, respectively that for the compound C_2 32, 128, 128, 128 and 64 $\mu\text{g mL}^{-1}$, respectively that for the compound C_3 32, 32, 64, 32 and

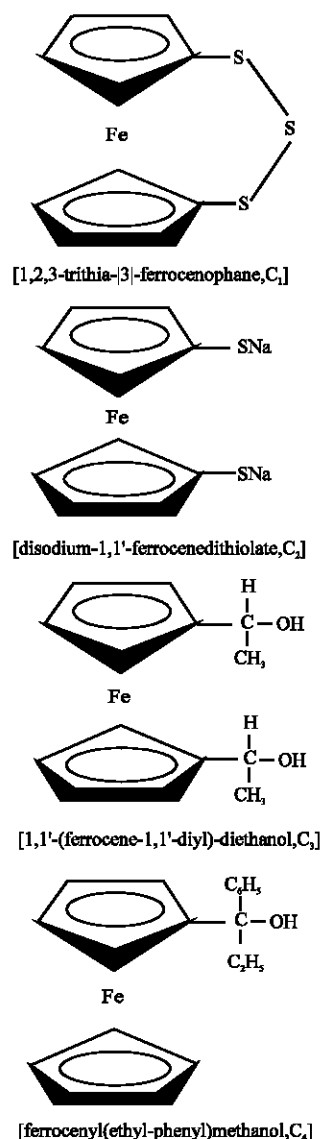


Fig. 1: Structure of investigated ferrocene derivative compounds

Table 1: *In vitro* antibacterial activity of the coordination complexes C_1 , C_2 , C_3 , C_4 and standard ciprofloxacin

Species	Diameter of zone of inhibition (in mm)									
	C ₁		C ₂		C ₃		C ₄		Ciprofl	
	30	200	30	200	30	200	30	200	30	
μg disc ⁻¹										
Gram positive bacteria										
<i>Bacillus subtilis</i>	00	16	00	11	09	21	00	18	31	
<i>Streptococcus β-haemolyticus</i>	00	17	00	13	11	22	00	17	27	
<i>Staphylococcus aureus</i>	00	14	00	10	00	16	00	14	28	
Gram negative bacteria										
<i>Pseudomonas aeruginosa</i>	00	16	00	14	00	16	09	16	28	
<i>Escherichia coli</i>	00	14	00	13	00	18	00	15	29	
<i>Klebsiella</i> species	00	16	00	15	09	19	00	17	29	
<i>Salmonella typhi</i>	09	17	00	11	00	19	00	14	26	
<i>Shigella dysenteriae</i>	09	18	00	12	00	15	00	18	27	

Table 2: Minimum Inhibitory Concentration (MIC) values of the complexes C₁, C₂ and standard amoxicillin

Test organisms	Minimum inhibitory concentration ($\mu\text{g mL}^{-1}$)				Ciprofloxacin
	C ₁	C ₂	C ₃	C ₄	
<i>Bacillus subtilis</i>	64	32	32	128	4
<i>Streptococcus</i> β -haemolyticus	64	128	32	128	2
<i>Escherichia coli</i>	64	128	64	64	2
<i>Salmonella typhi</i>	128	64	32	64	4
<i>Klebsiella species</i>	64	64	64	64	2

Table 3: *In vitro* antifungal activity of the complexes C₁, C₂, C₃, C₄ and standard nystatin

Species	Diameter of zone of inhibition (in mm)				
	C ₁	C ₂	C ₃	C ₄	Nystatin
	200 $\mu\text{g disc}^{-1}$				
<i>Candida albicans</i>	10	12	13	10	19
<i>Aspergillus niger</i>	00	10	17	10	21
<i>Aspergillus flavus</i>	00	00	15	00	20

Table 4: The results of cytotoxic effect of the complexes C₁, C₂, C₃, C₄ and standard bleomycin and gallic acid

Test samples	LC ₅₀ (ppm)	95% confidence limit (ppm)		Regression equation	χ^2 (df)
		Lower	Upper		
[1,2,3-trithia-3]-ferrocenophane, C ₁	52.471	15.45	178.13	Y = 3.191+1.120X	0.42(3)
[disodium-1,1'-ferrocenedithiolate, C ₂	41.138	16.70	101.32	Y = 3.191+1.120X	0.47(3)
[1,1'-(ferrocene-1,1'-diyl)-diethanol, C ₃	6.031	3.89	9.34	Y = 3.207+2.296X	0.03(2)
[ferrocenyl(ethyl-phenyl)-methanol, C ₄	105.838	19.16	584.33	Y = 3.023+0.976X	0.73(3)
Standard bleomycin	0.41	0.27	0.62	Y = 3.16+2.98X	0.62(2)
Gallic acid	4.53	3.33	6.15	Y = 3.93+1.62X	1.25(2)

64 $\mu\text{g mL}^{-1}$, respectively and that for the compound C₄ 128, 128, 64, 64, 64 $\mu\text{g mL}^{-1}$, respectively (Table 2). From the MIC results we can conclude that the ferrocene derivative compounds are not very active against the tested bacteria compared with the standard ciprofloxacin and this is probably due to different mechanism of actions of ferrocene compounds. In this present investigation we can conclude that the antibacterial activity of the ferrocene based complexes may differ for their legands.

Against the tested fungi the compound C₃ exhibited moderate activity in the concentration of 200 $\mu\text{g disc}^{-1}$ compared to the compounds C₁, C₂ and C₄ showed less activity (Table 3). The ferrocene based compound C₃ showed better activity against the *Aspergillus niger* but the other compounds C₁, C₂ and C₄ showed less activity against the tested fungi *Candida albicans* with the zone of inhibitions 10, 12 and 10 mm, respectively.

The LC₅₀ values of the ferrocene derivative compounds C₁, C₂, C₃ and C₄ were found to be 52.476, 41.138, 6.031 and 105.838 $\mu\text{g mL}^{-1}$, respectively (Table 4). The standard anticancer drug bleomycin gave its LC₅₀ value at 0.41 $\mu\text{g mL}^{-1}$. The lowest LC₅₀ value was found in case of the ferrocene based compound C₃ indicated its higher cytotoxicity than the compounds C₂, C₂ and C₄. The compound C₃ showed potent biocidal activity against brine shrimp nauplii as compared with the control DMSO and gallic acid, used as standard agent (Sarkar *et al.*, 1966).

DISCUSSION

On the ongoing research of exploring bioactive ferrocene derivative compounds, we were investigated four novel complexes and found interesting activities. The iron based complexes showed bactericidal as well as fungicidal activities. As the previous reports of the oxidative DNA damage property for the iron based complexes (Vijayalakshmi *et al.*, 2002; Joudah *et al.*, 2002), we may propose the probable mechanism of antimicrobial action for the novel complexes as to causing oxidative DNA damage but further investigations will be required to explore the exact mechanism of action for these complexes. Many coordination complexes of different transition metals had been reported for their antifungal activity (Islam *et al.*, 2002; Sultana *et al.*, 2003) but still we did not find sufficient data's for antifungal activity of iron complexes. It is therefore our interesting findings that the iron complexes may exhibit antifungal activity. The probable mechanism of fungicidal activity is the oxidative DNA damage as the previous reports (Vijayalakshmi *et al.*, 2002; Joudah *et al.*, 2002). Previously, many authors explored the cytotoxic activity of metal coordination complexes (Rho *et al.*, 2002; Treshchalina *et al.*, 1979; Kelland *et al.*, 1994). Cytotoxicity of cisplatin was found to be higher (LD₅₀ = 0.02 $\mu\text{g mL}^{-1}$) in previous literature against the L1210 tumor cells (Jung *et al.*, 2002). In the brine shrimp

lethality bioassay, we have found potent cytotoxic activity for the ferrocene based complex but further investigations taking human cancer cell line should be done to assess its cytotoxic potency. The cytotoxicity results of our present investigation suggest the previous reports of biocidal activities of iron based complexes (Vijayalakshmi *et al.*, 2002; Joudah *et al.*, 2002) but further studies will be required to assess its mechanism of cytotoxicity.

From the present findings of biocidal activity of the ferrocene complexes, we can conclude that the iron based complex may have some beneficial effect as anticancer agent which may come in the modern clinical trial.

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