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In vitro Antimicrobial Properties and Cytotoxic Activities of (Two Novel Deleted) Chromium Complexes

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Abstract: Objective: The aim of the present study was to investigate the antimicrobial and cytotoxic activities of two new chromium based coordination complexes $[Cr(Pht)_2(Cystine)_2]$ indicated C_1 and $[Cr(Suc)_2(Phenylamine)_2]$ indicated C_2, against Gram-positive and Gram-negative bacteria, fungi, and brine shrimp nauplii. Methods: *In vitro* antimicrobial susceptibility was determined as per National Committee for Clinical Laboratory Standards guidelines and serial dilution technique for the determination of minimum inhibitory concentration (MIC) of complexes and brine shrimp lethality bioassay for cytotoxicity assay for primary selection of the compounds as therapeutic agent. Results: The complex C_2 showed very high antibacterial activity at the concentration of 100 mg $disc^{-1}$ and gave its MIC values between 16-64 mg ml^{-1} against the tested microorganisms (both bacteria and fungi). The complexes gave comparatively better antibacterial activity against the Gram-positives than the Gram-negatives. C_1 and C_2 showed medium antifungal activity compared to standard nystatin against *Candida albicans*, *Aspergillus niger*, *Aspergillus fumigatus* and *Penicillium* species at the concentration of 200mg/disc. The LC_{50} values were calculated after probit transformation of the resulting data. All the complexes showed medium cytotoxic effect but between them C_1 having LC_{50} values of 3.54 mg ml^{-1} showed potent cytotoxicity compared with the reference standard Gallic acid and control DMSO. Conclusion: Our data shows that all the pathogenic microorganisms (Gram positive & negative bacteria and fungi) showed very high sensitivity with minimal cytotoxicity towards the complex C_2. But further work is necessary in order to explore the exact mechanism of their cytotoxic properties.

Keywords: Chromium complexes, antimicrobial activity, cytotoxic properties

INTRODUCTION

Coordination complexes of transition metal had been widely studied for their antimicrobial[1,2] and anticancer properties[3,4]. One of the most potent and effective antitumour agents was discovered in the last century serendipitously by Rosenberg *et al.,*[5]. Rosenberg and his coworkers synthesized several simple platinum complexes, among which cisplatin - $Pt(II)(NH_3)_2Cl_2$ - showed remarkable efficacy in inhibiting the growth of tumours in mice[6]. McGowan[7] reported the first clinical trials of cisplatin in 1971, with official approval being granted in the US in 1978. By 1983, cisplatin was the US's biggest selling antitumour drug and still one of the most widely used antitumour drugs. It is one of the most effective drugs for treating testicular, ovarian, bladder and neck cancers. Over the past 30 years, platinum-based drugs notably cisplatin and carboplatin have dominated the treatment of various cancers by chemical agents. Despite the success of cisplatin, however, it lacks selectivity for tumour tissue, which leads to severe side effects including renal impairment, neurotoxicity and ototoxicity. Various tumor cell lines are now growing resistance to cisplatin e.g., acquired cisplatin resistance in some preclinical tumor models[8]. The scientists are now engaged to explore other transition metal complexes as antitumour agents and considerable results have brought through the discovery of titanium based complexes[9] and other transition metal based complexes[10,11]. Among the other transition metal complexes the titanium complex, titanocene dichloride ($TiCp_2Cl_2$) is the only metallocene-based compound to have entered clinical trials for its potent and broad spectrum activity in mammalian tumors[7]. Compared to standard antineoplastic agents such as cisplatin,

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doxorubicin, mitoxantrone and vinblastine, titanocenedichloride was found to exhibit higher cytotoxicity in renal cell carcinoma[9]. Recently some derivatives of titanocenedichlorides showed enhanced anti-cancer activity[12]. Chromium complexes have also been reported for their potent cytotoxic activity[11]. Therefore, it is of our interest to study the cytotoxic and antimicrobial properties of two novel coordination complexes of chromium to assess their biological potency.

MATERIALS AND METHODS

Preparation of Complexes: The complexes were synthesized according to the following procedure: The aqueous solution of chromium (III) chloride and of amino acids containing minimum amount of KOH (to make soluble) were mixed in a molar ratio of 1:2 and then allowed to stand for about ten minutes. Two moles of imide slats (potassium phthalimide or potassium succinimide) were then added. To get the precipitates of complexes, the mixture were then heated at 60°C for about twenty five minutes and then allowed to stand for ten minutes. The precipitates formed were removed by filtration, washed several times with distilled water and finally with alcohol and dried in a vacuum desiccator over anhydrous $CaCl_2$. The prepared complexes were characterized by IR, UV, magnetic moment, melting point and conductivity measurement[13].

According to the following equations the complexes of the chromium (III) were obtained.
For phthalimide based complexes

$$H\text{-}Pht + KOH \rightarrow K\text{-}PHT + H_2O$$

$$CrCl_3 + A\text{-}H + K\text{-}Pht \rightarrow K[Cr(Pht)_2(A)_2]$$

And for succinimide based complexes-

$$H\text{-}Suc + KOH \rightarrow K\text{-}Suc + H_2O$$

$$CrCl_3 + A\text{-}H + K\text{-}Suc \rightarrow K[Cr(Suc)_2(A)_2]$$

Where:

Pht = anions of phthalimide
Suc = anions of succinimide
A = amino acids e.g., phenylalanine and cystine.

Antibacterial Screening: *In vitro* antibacterial screening is generally performed by disc diffusion method[14] 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[15]. The antibacterial activity of the complexes C_1, and C_2 was determined at a concentration of 30 µg/disc and 200 µg/disc against four Gram-positive (*Bacillus subtilis, Streptococcus b-haemolyticus, Staphylococcus aureus* and *Bacillus megaterium*) and four Gram-negative (*Escherichia coli, Shigella sonnei, Shigella dysenteriae* and *Shigella shiga*) bacteria. The diameters of the zone of inhibition produced by the compounds were compared with the standard antibiotic (Kanamycin, 30 µg/disc). The experiments were performed at four times to minimize the error.

Growth Media and Conditions: Nutrient broth was used as liquid culture of all the tested bacteria and is used in the minimum inhibitory concentration determining experiments. Potato dextrose agar (PDA) media was prepared in the lab to maintain the fungal growth. Antifungal activity of the complexes was done of PDA media spreading with fungal spores and kept at 28 ºC for about 72 hours. For PDA preparation 20 gm Potato was extracted with distilled water 100 ml at 100 ºC for 1 hour and it was then filtered off by cotton filter. The potato juice (100 ml) was then mixed with 2 gm Dextrose and 1.5 gm agar and finally the p^H of the prepared media (PDA) was adjusted at 7.00.

MIC Measurements: A current definition of the Minimum Inhibitory Concentration, MIC, is ``the lowest concentration which resulted in maintenance or reduction of inoculum viability''[16]. 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[17]. Serial dilution technique[15] was applied for the determination of minimum inhibitory concentration of complexes. Four bacterial species (*Bacillus subtilis*, *Eschericia coli Salmonella typhi* and *Shigella dysenteriae*) and three fungal species (*Candida albicans, Aspergillus flavus* and *Aspergillus niger*) were used. DMSO was used for our experiments made dilutions of the coordination complexes under test in MIC determination. Bacteria were incubated on nutrient agar slants for 18 h at 30 °C. MIC tests were run with the third subculture of bacteria and samples were taken during the exponential phase of bacterial growth.

Bacterial and fungal inocula were prepared at 5×10^6 - 5×10^7 cfu/ml. Final adjustment were made using optical density measurement for bacteria (absorbance 0.05 at a wavelength of 660 nm).

Collection of the Bacterial and Fungal Species: The bacterial species used in this experiment were *Bacillus subtilis* (QL-40), *Streptococcus b-haemolyticus* (ATCC-12873), *Staphylococcus aureus* (ATCC-25933), *Bacillus megaterium* QL-38), *Escherichia coli* (ATCC-25922), *Shigella sonnei* (AJ-8992), *Shigella dysenteriae* (AL-35587) and *Shigella shiga* (ATCC-26107) all of which were collected from the Institute of Nutrition and Food Sciences (INFS), Dhaka University, Bangladesh.

Tested fungi *Candida albicans* (ATCC 10231), *Aspergillus fumigatus* (ATCC 1028), *Aspergillus niger* (CCRC 31494) and *Penicillium* species were collected from the Institute of Biological Sciences (IBSc), Rajshahi University, Bangladesh, from their stock culture

Antifungal Screening: The antifungal activity of the complexes were tested by disc diffusion method[14] against the four pathogenic fungi *Candida albicans*, *Aspergillus niger, Aspergillus fumigatus* and *Penicillium* species at a concentration of 200 µg/disc for each. The media used in this respect was potato dextrose agar (PDA). The activity was determined after 72 hours of incubation at room temperature (25 ºC).

Cytotoxicity Bioassay: Brine shrimp lethality bioassay[18,19,20] is a recent development in the assay procedure of bioactive compounds which indicates cytotoxicity as well as a wide range of pharmacological activities (e.g. anticancer, antiviral, insecticidal, pesticidal, AIDS, etc.) of the compounds. In this method, the eggs of the brine shrimp, *Artemia salina* Leach, were collected from an aquarium shop (Dhaka, Bangladesh) and hatched for 48hr to mature shrimp. 38g of sea salt was weighed, dissolved in one liter of distilled water, filtered off and was kept in a small tank. The eggs were then added to the divided tank. Constant oxygen supply was provided and temperature (37±1ºC) was maintained for 48hr to hatch and mature the shrimp called as nauplii (larvae). The test sample extract was prepared by dissolving them in DMSO (not more than 50ml in 5ml solution)plus sea water (3.8% NaCl in water) to attain concentrations - 5,10,20,40 and 80mg/ml. A vial containing 50ml DMSO diluted to 5ml was used as a control. Then about 10 brine shrimp nauplii were applied to each of all experimental vials and control vial. The number of the nauplii that died after 24hr was counted. The resulting data were transformed to the probit analysis[21] for the determination of LC_{50} values for the complexes.

Statistical Analysis: Statistical analyses of the antibacterial and antifungal activities of seven novel thiocyanato complexes with different concentrations of each (30 and 200 µg/disc) was performed using Kruskal-Wallis test[22]. Individual antibacterial and antifungal activity differences of the tested complexes ($C_1$and C_2) was examined using post hoc Nemenyi's test following Kruskal-Wallis test. A significance level of 5 % was considered as significance ($P< 0.05$) in all cases. Probit analysis[21] was used to determine the LD_{50} values from the mortality data using Probit software. The cytotoxicity of the novel thiocyanato coordination complexes was compared with the standard gallic acid and also with the anticancer agent bleomycin. Determination of LD_{50} by probit analysis allowed the ranking of these coordination complexes with respect to their biocidal activity.

RESULTS AND DISCUSSIONS

Results: At concentration of 100 µg/disc the complexes C_1 and C_2 showed a remarkable antibacterial activity against the tested Gram positive and Gram negative bacteria (Table 1). The antifungal activities of the metal complexes and standard nystatin (N-50 mg/disc) were determined at the concentration of 200 mg/disc against four pathogenic fungi. It was found that the

Table 1: In vitro antibacterial activity of the coordination complexes C_1, C_2 and standard Kanamycin.

µg/disc	Diameter of zone of inhibition (in mm)				
	C_1		C_2		Kanamycin
	30	100	30	100	30
Gram positive bacteria					
Bacillus subtilis	10	15	10	19	22
Streptococcus b-haemolyticus	00	17	11	18	20
Staphylococcus aureus	00	15	09	17	23
Bacillus megaterium	11	17	12	20	27
Gram negative bacteria					
Shigella shiga	09	13	11	18	19
Escherichia coli	00	11	09	16	21
Shigella dysenteriae	00	12	10	16	20
Shigella sonnei	08	14	11	18	22

Table 2: Antifungal activities of the complexes C_1, C_2 and standard Nystatin.

	Diameter of zone of inhibition (in mm)		
	C_1	C_2	Nystatin
µg/disc →	200	200	50
Candida albicans	15	18	19
Aspergillus niger	10	16	21
Aspergillus fumigatus	13	20	20
Penicillium species	12	19	25

Table 3: Minimum Inhibitory Concentration (MIC) values of complexes C_1 and C_2.

	Minimum inhibitory concentration (mg ml^{-1})			
Test organisms	C_1	C_2	Kanamycin	Nystatin
Bacillus subtilis	64	16	4	-
Bacillus megaterium	64	32	4	-
Shigella sonnei	32	32	8	-
Shigella shiga	64	32	4	-
Candida albicans	64	32	-	2
Aspergillus fumigatus	32	32	-	4
Aspergillus niger	32	64	-	2

metal complex C_2 was shown greater activity than C_1 against all of the pathogenic fungi. Table 2 showed that the complex C_2 was noticeable active against the tested fungi at concentration of 200µg/disc with comparing the standard nystatin. The MIC values of the complexes against *Bacillus subtilis, Bacillus megaterium, Shigella sonnei, Shigella shiga, Candida albicans , Aspergillus fumigatus* and *Aspergillus niger* were shown in Table 3. The MIC values of the complex C_1 was higher than C_2 against the tested

Table 4: The cytotoxic effect of complexes C_1, C_2 and standard bleomycin and gallic acid

Test samples	LC_{50}	95% confidence limit (ppm)		Regression equation	c^2 (df)
	(ppm)	Lower	upper		
C_1	3.54	2.08	6.02	Y=3.98+1.85X	3.38(2)
C_2	6.49	4.15	10.15	Y=3.17+2.27X	0.35(2)
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)

bacteria which indicated that the complex C_2 was more active against the bacteria and fungi. The mortality rate of brine shrimp nauplii was found to increase with increasing the concentration of complexes. Table 4 summarizes that the LC_{50} values of the complexes C_1 and C_2 were found at 3.54 and 6.49 µg/ml (ppm), respectively. The standard anticancer drug bleomycin gave its LC_{50} value at 0.41 µg/ml. The lowest LC_{50} value at 3.54 ppm was found in case of complex C_1 which was indicative of its potent cytotoxicity than the other coordination complex C_2 in this experiment.

Discussion: It was found that the metal complex C_2 was more active than C_1 against all of the test bacteria. The newly synthesized complex C_2 displayed poor antibacterial activity at the concentration of 30 µg/disc, but gave promising activity at concentrations of 100µg/disc against the tested bacteria in comparison with the standard kanamycin. In the present investigation we found that the complexes showed comparatively better antibacterial activity against the Gram positive bacteria than the Gram negative bacteria. Many authors reported antibacterial activity of different transition metal complexes[23,24] and our present findings supported the previous investigations.

The MIC values of this complex against the tested organisms indicated their noticeable antibacterial and antifungal potencies compared with standard antibiotic, kanamycin and nystatin respectively. For C_2 the MIC values were 32, 32, 32, 64, 64 32, & 64 µg/ml, respectively against the organisms; whereas the standard compounds showed MIC values between 2-8 µg/ml which was indicative of their potent antimicrobial properties than the complexes. The mechanism of biocidal activity of these coordination complexes may be due to oxidative DNA damage as the previous reports[11]. The different antibacterial activity of the complexes indicated their different mechanism of biocidal property and further studies are required to explore the exact mechanism of antibacterial potency[25].

The maximum zone of inhibition against *Aspergillus fumigatus* and *Penicillium* species were found to be 20 and 18 mm respectively, for the complex C_2 and C_1 which were near to the zone of inhibition of 10-15 mm where as 20 and 25 mm for standard, nystatin. Different metal coordination complexes have been previously reported for their antifungal properties[1,24] which supports our present findings.

Chromium based complexes have been reported previously for their potent cytotoxic properties than platinum based complexes[9] and our present findings also support the previous investigations as the chromium based complex C_1 (LC_{50}= 3.54 ppm) showed more cytotoxicity than the complex C_2 (LC_{50}= 6.49 ppm). Compared to standard antineoplastic agents such as cisplatin, doxorubicin, mitoxantrone and vinblastine, titanocenedichloride (titanium complex) was found to exhibit higher cytotoxicity in renal cell carcinoma[9]. The titanium based complexes was also found to exhibit more effective in mammalian cancer model than cisplatin[26]. Therefore it is of our interest to explore some novel transition metal based complexes as potent cytotoxic agents which might come as potent anticancer agent in clinical trials. In the present investigations we found a novel chromium based complex C_2 with potent antimicrobial agent and had moderate cytotoxicity.

Cytotoxic properties of coordination complexes had been previously reported by many authors[27,28,29] and our present findings also displayed the similar type of properties for the newly synthesized chromium complexes. The different LC_{50} values for the chromium complexes indicated the different mode of actions of their cytotoxicity.

Conclusion: It was concluded from the results that between the tested complexes, the complex C_2 possesses very high antimicrobial activity with a minimum inhibitory concentration and medium cytotoxicity. Further investigations are required to explore the exact mechanism of their cytotoxic properties which may be helpful for to explore new type of potent cytotoxic agent(s) with the hope of adding new and alternative chemotherapeutic agent(s) in clinical implications.

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