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Biological Screening of a Novel Nickel (II) Tyrosine Complex

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A newly synthesized Nickel (II) tyrosine complex was screened as potential antimicrobial agent against a number of medically important bacteria (*Bacillus subtilis, Streptococcus β-haemolytica, Escherichia coli, Shigella dysenterae*) and fungi (*Aspergillus fumigatus, Candida albicans, Aspergillus niger, Aspergillus flavus, Penicillium* sp.) strains. were used for antifungal activity. The antimicrobial activity was evaluated using the Agar Disc method. Moreover, the minimum inhibitory concentration of the complexes was determined against the same pathogenic bacteria and the values were found between 4~64 $\mu g\ ml^{-1}$. Brine shrimp bioassay was carried out for cytotoxicity measurements of the complexes. The LC_{50} values were calculated after probit transformation of the resulting mortality data and found to be 6 $\mu g\ ml^{-1}$.

KEYWORDS: Antimicrobial activity, Cytotoxicity, Nickel (II) tyrosine complex, Pathogens

The discovery and development of antibiotics are among the most powerful and successful achievements of modern science and technology for the control of infectious diseases. However, the increasing microbial resistance to antibiotics in use, nowadays necessitates the search for new compounds with potential effects against pathogenic microbes. The most spectacular advances in medicinal chemistry have been made when metal complexes heterocyclic compounds played an important role in regulating biological activities. Extensive investigations in the field of metal complexes have been reported (Sheikh *et al.*, 2004; Cik *et al.*, 2001).

Due to the great flexibility and diverse structural aspects of metal complexes, a wide range of these compounds have been synthesized and their complexation behavior studied (Ruiz *et al.*, 1995; Douglas *et al.*, 1998).

Many drugs possess modified pharmacological and toxicological properties when administered in the form of metallic complexes. Probably the most widely studied cation in this respect is Cu^{2+}, since a host of low-molecular-weight copper complexes have been proven beneficial against several diseases such as turberculosis, rheumatoid, gastric ulcers, and cancers (Ruiz *et al.*, 1995). Several binary and ternary complexes of Pd (II) and Pt (II) were reported with nucleic acid constituents, amino acids and other nitrogen containing ligands (Douglas *et al.*, 19984). Metal complexes of semicarbazones and thiosemicarbazones have aroused considerable interest in view of their industrial and biological importance (Singh *et al.*, 2005). Many of these compounds possess a wide spectrum of medicinal properties, including activity against tuberculosis, leprosy, and bacterial and viral infections. They have also been found to be active against influenza, protozoa, smallpox, psoriasis, rheumatism, trypanosomiasis, coccidiosis, malaria and certain kinds of tumors and have been suggested as possible pesticides and fungicides. Their activity has frequently been thought to be due to their ability to chelate trace metals (Chandra and Gupta, 2005). Moreover, Nickel (II) complex derived from aminosugars showed effective antifungal activity against pathogenic Yeast, *Candida albicans* with MIC, this complex act as inhibitor for chitinase (chitin-degradation enzyme) enzyme of *C. albicans* (Yano *et al.*, 1998). In addition, Nickel (II) complex of 5-methyl 2-furfural thiosemicarbazonehave carried out *in vitro* for antifungal activity on human pathogenic fungi, *Aspergillus fumigatus* and *Candida albicans*, and *in vivo* for toxicity on mice (Jouad *et al.*, 2001). Transition metal coordination complexes have now been widely studied for their antimicrobial and anticancer properties (Kamalakannan and Venkappayya, 2002). The scientists are now engaged to explore other transition based complexes and other complexes (Quievryn *et al.*, 2003; Shivankar and Takkar, 2003).

In the continuation of this discovery present studies synthesized a new Ni (II) tyrosine complex for the first time and the antibacterial and antifungal effect of this

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complex against different bacteria and fungus. In addition, cytotoxic effect of the complex was also evaluated.

Materials and Methods

Synthesis of Ni (II) tyrosine complex. Saturated solution of amino acid (tyrosine) and Nickel acetate were made with distilled water. The solutions were then mixed together in the ratio 1 : 2 (Amino acid: Ni (II) acetate). The pH of the solution was adjusted to 6 and allowed to stand on water bath at 40°C for 6 hrs. The solution was then cooled to room temperature. The crystal of Ni (II) tyrosine complex was then separated from the solution and washed with distilled water and dried in a desiccator. The product was crystalline, green and soluble in water and alcohol and free from starting materials (Checked by TLC). Yield: 0.0297 g, 91%.

Antibacterial screening. Two gram positive such as *Bacillus subtilis* (ATCC 6633), *Streptococcus β-haemolytica* and two gram negative namely *Escherichia colli* (ATCC 14169), *Shigella dysenterae* (AL 35587) bacteria were selected for this study. Nutrient agar was used as antibacteriological media. Antibacterial potency of Ni (II) tyrosine complex was measured against all the tested bacteria according to the standard disc diffusion method where air dried sterile Whatman filter paper discs (6 mm diameter) with centers of at least 24 mm apart were deposited on nutrient agar plate using aseptic technique.

Bacterial inoculum containing approximately, 10^4~10^6 colony forming units (CFU) ml^{-1} were spread on the surface of nutrient agar. The test complex at doses 150, 210 and 30 μg/disc was added into three discs. The forth disc was supplemented with reference drug Kanamycin at dose 30 μg/disc serving as positive control. The plates were incubated immediately at 37°C for 14~19 hrs. Activity was determined by measuring the diameter of zones (mm) showing complete inhibition. Growth inhibition was calculated with respect to positive control.

Minimum inhibitory concentration (MIC) determination. MIC of the compound is defined as the lowest concentration of that compound in a medium without visible growth of the test organisms. MIC of the complexes was determined against two gram positive (*B. subtilis*, *S. β-haemolytica*) and two gram negative (*E. coli*, *S. dysenterae*) pathogenic bacteria by serial dilution technique (Reiner, 1982).

The media used in this respect was nutrient broth media. Decreasing concentration of Ni (II) tyrosine complex was prepared in serial two fold dilution using the stock solution (1.024 mg ml^{-1}). 10 μl of bacterial suspension (10^7 cells ml^{-1}) was inoculated into all of the test tube. After incubation for 24 hrs at 37°C, the test tubes were observed for no growth and growth of the used bacteria.

Antifungal assay. Two animal fungi (*Aspergillus fumigatus*, *Candida albicans*) and three plant fungi (*Aspergillus niger*, *Aspergillus flavus*, *Penicillium* sp.) were selected for this study. Seaboard dextrose agar (SDA) for animal fungi and Potato dextrose agar (PDA) for plant fungi were used for fungal growth media. Antifungal activity of Ni (II) tyrosine complex was observed against all of the test microorganisms according to the standard disc diffusion method (Beur *et al.*, 1966), where air dried sterile Whatman filter paper discs (6 mm diameter) with centers of at least 24 mm apart were deposited on growth media in plate using aseptic technique (Patel *et al.*, 1993). Fungal inoculum containing approximately 10^5 spores/*ml* were spread on the surface of growth media. The test compound at doses 250 and 500 μg $disc^{-1}$ and known drug Fluconazole INN at dose 250 μg $disc^{-1}$ serving as positive control were applied into three discs respectively. Plates were kept at low temperature (4°C) for 24 hrs to allow maximum diffusion then incubated immediately at 18~27°C for 5~7 days. Activity was confirmed by measuring the diameter of zones (mm) showing complete inhibition. Growth inhibition was calculated with respect to positive control.

Cytotoxicity bioassay. Brine shrimp lethality bioassay is a recent development in the assay procedure of bioactive compound, which indicates cytotoxicity as well as a wide range of pharmacological activities (such as anticancer, antiviral, insecticidal, pesticidal, AIDS etc) of the compounds (Zahan *et al.*, 2004). Here Twelve vials were taken (two vials for each concentration) for this study. Five ml of sea water was given to each of the vial. 2, 4, 8, 16, 20 μg ml^{-1} solution of Ni (II) tyrosine complex were transferred to 10 vials and 2 vials were used as control. With the help of pasteur pipette 10~11 living shrimps were inoculated into each of the vial.

After 24 hrs of incubation, each vial was observed using a magnifying glass and the number of survivors in each vial was counted and noted. From the data, % of mortality was calculated and plotted against Log dose (log C, means Log of dose concentration, that means 'effective concentration' or 'effective dose' which is the concentration of test materials in water, soil or sediment (e.g. mg/*l* or mg/kg) or dose of test material (e.g. mg/Kg-body weight in an avian bolus study) that causes x% change in response (e.g. mortality, immobility) during a specified time interval. This corresponds to an effect predicted on x% of the test organisms at a given concentration. This parameter is estimated by concentration-response modelling). From the graph LC_{50} values of the complexes were determined using probit analysis (Finncy, 1971).

Table 1. Antibacterial activity of Ni (II) tyrosine complex

Test bacteria	Mean diameter of inhibition[a] (mm)		
	Ni (II) tyrosine complex		Kanamycin
	150 μg disc^{-1}	210 μg disc^{-1}	30 μg disc^{-1}
Gram positive			
Bacillus subtilis	[a]11	15	22
Streptococcus β-haemolytica	12	16	21
Gram negative			
Escherichia coli	10	20	20
Shigella dysenterae	14	24	22

Table 3. Antifungal activity of Ni (II) tyrosine complex

Test Fungus	Mean diameter of inhibition[a] (mm)		
	Ni (II) tyrosine complex		Fluconazole INN
	250 μg disc^{-1}	500 μg disc^{-1}	250 μg disc^{-1}
Animal fungi			
Aspergillus fumigatus	13	21	0
Candida albicans	20	28	0
Plant fungi			
Aspergillus niger	18	23	20
Aspergillus flavus	15	29	21
Penicillium sp.	11	13	16

Results

Antibacterial activity. Antibacterial activity of Ni (II) tyrosine complex was determined by using doses 150 and 210 μg disc^{-1} and results were shown in (Table 1). Ni (II) tyrosine complex showed remarkable sensitivity against used bacteria for all doses and results were compared with known antibacterial drug kanamycin (30 μg disc^{-1}). Ni (II) tyrosine complex at dose 150 μg disc^{-1} showed small diameter of zone of inhibition such as 11, 12, 10, 14 mm against *Bacillus subtilis*, *Streptococcus β-haemolytica*, *E. coli* and *Shigella dysenterae* respectively as compared with kanamycin at dose 30 μg disc^{-1} which were 22, 21, 20 and 22 mm against the same bacteria respectively. Whereas at dose 210 μg disc^{-1} of compound showed moderate activity (15, 16, 20 and 24 mm) against the same bacteria as compared with kanamycin (30 μg disc^{-1}).

Minimum inhibitory concentration (MIC). MIC is the lowest amount of drug at which it is able to inhibit the growth of specified microorganism. MIC of Ni (II) tyrosine complex against *B. subtilis*, *S. β-haemolytica*, *E. coli* and *S. dysenterae* (Table 2) were observed no growth in the test tube at 1024, 512, 256, and 128 $\mu g\ ml^{-1}$ respectively.

Antifungal activity. Antifungal activity Ni (II) tyrosine complex was investigated by using doses 250 and 500 μg disc^{-1} and results were shown in Table 3. Nickel (II) tyrosine complex showed moderate activity against used fungus for all doses and compared with known antifungal drug Fluconazole INN (250 μg disc^{-1}). At the dose of 250 and 500 μg disc^{-1} of Ni (II) tyrosine complex, the diameter of zone of inhibition were evaluated as 13, 20, 18, 15 & 11 mm and 21, 28, 23, 29 & 13 mm against *A. fumigatus*, *C.albicans*, *A. niger*, *A. flavus* and *Penicillium* sp. respectively, where the diameter of zone of inhibition of Fluconazole INN (250 μg disc^{-1}) was found to be zero for *A. fumigatus*, *C. albicans*, but in case of *A. niger*, *A. flavus* and *Penicillium* sp. the zone was found to be 20, 21 and 16 mm respectively.

Cytotoxic activity. To find out the effect of Ni (II) tyrosine complex on the mortality of brine shrimp nauplii, median lethal concentration of brine shrimp (LC_{50}) was calculated (Table 4) and it was found to be 6 $\mu g\ ml^{-1}$. The plot of % of mortality versus log of Ni (II) tyrosine complex showed an approximate linear correlation between them.

Discussion

Antibacterial activity. Standard drug kanamycin (30 μg disc^{-1}) showed the diameter of zone of inhibition from 20~22 mm against used bacteria. On the other hand, the diameter of zone of inhibition measured from 15~24 mm (equivalent to kanamycin) of Ni (II) tyrosine complex against used bacteria at the dose of 210 μg disc^{-1} (seven times increase concentration of kanamycin). This sug-

Table 2. MIC of Ni (II) complex against gram positive and gram-negative bacteria

Test bacteria	Concentration of Ni (II) complex ($\mu g\ ml^{-1}$)								
Gram positive bacteria	1024	512	256	128	64	32	16	8	4
Bacillus subtilis	–	–	–	–	+	+	+	+	+
Streptococcus β-haemolytica	–	–	–	–	+	+	+	+	+
Gram negative bacteria									
Escherichia coli	–	–	–	+	+	+	+	+	+
Shigella dysenterae	–	–	–	–	+	+	+	+	+

(+) = Growth and (–) = No growth.

Table 4. Effect of Ni (II) tyrosine complex on brine shrimp lethality bioassay

Concentration ($\mu g\ ml^{-1}$)	Log of concentration	No. of nauplii taken	No. of nauplii alive	No. of nauplii died	% of mortality	LC_{50} ($\mu g\ ml^{-1}$)
2	0.30	12	9	3	25	
4	0.60	10	7	3	30	
8	0.90	10	5	5	50	6
16	1.20	11	2	9	81.81	
20	1.30	10	1	9	90	

gested that the antibacterial activity of Ni (II) tyrosine complex lower than kanamycin. Metal complexes have been reported for their antibacterial activity (Chaudhary *et al.*, 2003).

Moreover, many authors also reported antibacterial activity of other transition metal complexes and our present findings also supported the previous results of antibacterial activity (Rehman *et al.*, 2005; Patel *et al.*, 2006).

So, the antibacterial activity of the complex may be due to the metal nickel. Further studies were needed to explore the mechanism of antibacterial activity of this tyrosine derivative compound.

Minimum inhibitory concentration (MIC). Earlier the minimum inhibitory concentration of a novel quaternary Copper (II) complex was determined against some pathogenic bacteria (Wu *et al.*, 2003). Moreover, data in Table 2 show that the MIC of Ni (II) complex against gram positive and gram-negative bacteria has also a significant value. It is important to note that the metal chelates exhibit more inhibitory effects than the parent ligands. The increased lipophilic character of these complexes seems to be responsible for their enhanced biological potency. It may be postulated that these compounds deactivate various cellular enzymes, which play a vital role in various metabolic pathways of these microorganisms. It has also been proposed that the ultimate action of the toxicant is the denaturation of one or more proteins of the cell, which, as a result, impairs normal cellular processes (Singh *et al.*, 2005).

Antifungal activity. The antifungal activity of other transition metal complexes also reported earlier (Chohan *et al.*, 2006). Our present findings support the previous results. It is observed from these studies that metal chelates have a higher activity than the free ligand. Such increased activity of the metal chelates can be explained on the basis of Overtone's concept and chelation theory (Raman *et al.*, 2003). According to Overtone's concept of cell permeability the lipid membrane that surrounds the cell favours the passage of only lipid soluble materials due to which liposolubility is an important factor that controls antifungal activity. On chelation, the polarity of the metal ion is reduced to a greater extent due to the overlap of the ligand orbital and partial sharing of the positive charge of the metal ion with donor groups. Further, it increases the delocalisation of *p*-electrons over the whole chelate ring and enhances the lipophilicity of the complex. This increased lipophilicity enhances the penetration of the complexes into lipid membranes and blocking of metal binding sites on the enzymes of the microorganism.

Cytotoxic activity. Many authors explored the cytotoxic properties of ferrocene derivatives compounds and found higher activities in case of metal complexes (Ferie-Vidovic and Poljak-Blazi, 2000; Top *et al.*, 2003). Depending upon our observation, the present observation may be concluded that, the tested Nickel (II) tyrosine complex has strong cytotoxic activity but this investigation is a primary one and farther tests are required to investigate its actual mechanism of cytotoxicity and its probable effects on higher animal model and on cancer cell line. It suggests that the complexes can be used as potent cytotoxic agents with the hope of adding arsenal of weapons used against the fatal disease cancer.

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