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ISSN: 2278-1862

Journal of Applicable Chemistry

2018, 7 (5): 1395-1403

(International Peer Reviewed Journal)

Studies of Nickel Phenothiazine Oleate as Biologically Potent Agent

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Accepted on 24th August, 2018

ABSTRACT

The solid surfactant of nickel has been derived from oleic acid, and a complex of nickel with tricyclic ligand like phenothiazine containing nitrogen as donor atom has been synthesized and characterized by elemental analysis, IR, NMR and ESR spectroscopy and magnetic moment studies. The magnetic moment studies suggested the dimeric nature of complexes. Spectral studies confirm that complexation has taken place successfully. The results indicate that complex has nonelectrolyte nature and possess octahedral geometry. The antifungal activities of this ecofriendly complex have been studied against Candida, A. niger, T. ressi and Penicillum. Antibacterial studies also done against Staphylococcus, Streptomyces, E.coli and Bacillus.

Graphical Abstract

T. ressi *Candida* *Penicillum* *A. niger*

Antifungal activity of Ni-complex on *T.ressi, Candida, Pencillium* and *A. niger*

Keywords: Phenothiazine, Nickel oleate, Antifungal, Antibacterial, Octahedral geometry.

INTRODUCTION

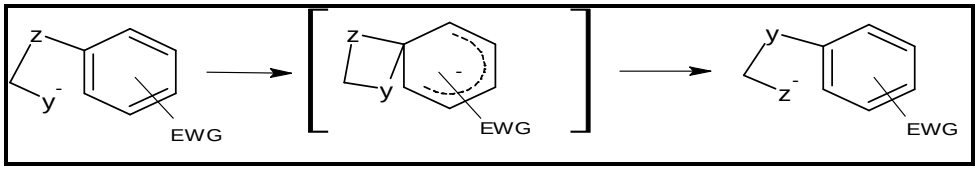
The chemistry of nitrogen – sulphur heteroatomic aromatic compounds like phenothiazine containing two benzene rings linked in a tricyclic system is becoming more popular as an area of research [**1**]. A change in substitution on phenothiazine nucleus causes a great difference in their biological activities [**2**]. Phenothiazine derivatives have been reported some discrete biological activities, such as antimicrobial [**3**], anti-inflammatory [**4, 5**], antitubercular [**6**], antitumor [**7, 8**] etc. Also, some of phenothiazine derivatives have been reported to exhibit anticancer activities [**9-11**] and inhibits intracellular replication of HIV [**12, 13**].

New classes of antifungal drugs are an important clinical need. Eilan *et al.* [**14**] first discovered the activity of phenothiazine against *C. albicans* and *C. neoformans*. Recently, several groups have shown that phenothiazine and it's derivatives are highly active against medically important molds such as *Zygomycetes* and *Aspergillus* [**15**].

In this present investigation we studied the antifungal character of phenothiazine metal (Ni) complex by agar well diffusion method. This method is widely used to evaluate the antimicrobial activities [**16, 17**]. In this method agar plates are inoculated with a standardized inoculum of the test micro-organism; by spreading a volume of the microbial inoculum over the entire agar surface. Then, a hole with a diameter of 6 to 8 mm is punched aseptically with a sterile cork borer or a tip, and a volume (20-100 sec) of the antimicrobial agent at desired concentration is introduced into the well. Then, agar plates are incubated under suitable condition depending upon the test microorganism. The antimicrobial agents diffuse in the agar medium and inhibit the growth of the microbial strain tested. Results of antifungal tests and antibacterial tests are subjected to statistical studies by using ANOVA technique.

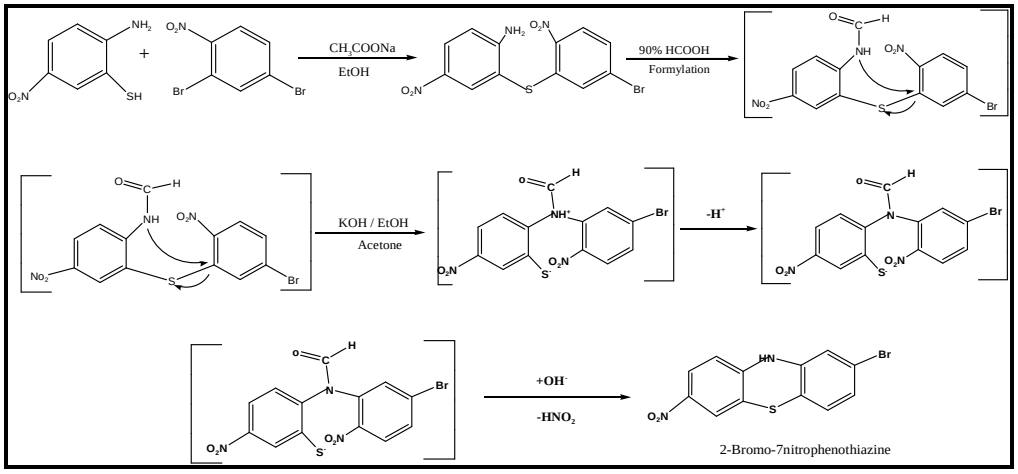
MATERIALS AND METHODS

Smiles rearrangement pathway is used to produce our ligand which is substituted phenothiazine **[18]**. Smiles rearrangement is an intramolecular nucleophilic aromatic substitution reaction which was discovered in 1931 by Samuel Smiles [**19**]. In this rearrangement a nucleophile Y displaces an aromatic nucleophile Z under a basic condition (Scheme 1).



Scheme 1. Smiles Rearrangement

In this present work reaction of 2,4-dibromo-nitrobenzenewith nitro substituted 2-amino benzene-thiol carried out to prepare bromo substituted phenothiazine. The reaction involves acidic medium to start and then we use KOH for basic medium for Smiles rearrangement as shown below scheme 2.



Scheme 2. Synthesis of Phenothiazine

Preparation of Substituted phenothiazine: To prepare bromo substituted phenothiazine at first, we synthesized nitro substituted benzenethiol and react it with meta dibromo nitrobenzene in alkaline medium. In present work we use 3.32 g (0.01 mole) of benzenethiol, 0.4 g (0.01 mole) sodium hydroxide and 2.02 g (0.01 mole) bromonitrobenzene in absolute alcohol (25 mL) and this mixture was refluxed for 1-2 h. After it mixture was cooled and filtered. Residue was washed with hot water and 80% ethanol and crystallized from acetone.

Preparation of Nickel surfactant: Nickel oleate was prepared by mixing one gram of oleic acid into 25 mL ethyl alcohol, shake the mixture in hot water bath about 50°C and then add one drop of phenolphthalein. Prepare a saturated solution of KOH in another beaker and add it into the oleic acid solution drop by drop until the light pink color appears.

Now again in another beaker prepare a saturated solution of $NiCl_2$ (about 3-4 g in 5 mL of water) and mix it into the above solution with constant stirring till a colored soap (blue color for Nickel oleate soap) is formed. Filter and wash it with warm water and 10% ethyl alcohol, then dried and recrystallized with hot benzene.

Preparation of Nickel complex with 2-bromo-7-nitrophenothiazine: The complex of nickel oleate and phenothiazine was prepared by adding 0.621 g (0.001 mole) nickel oleate with 0.646 g (0.002 mole) substituted phenothiazine in 25-30 mL ethyl alcohol and the mixture was refluxed for about two hours with constant stirring. After cooling the solid separate out was filtered, dried and recrystallized with hot benzene.

Antimicrobial Studies: Chemically derived complex of nickel oleate with substituted phenothiazine is being subjected to antimicrobial studies. There are four bacterial and fungal strains were selected for this peruse.

Micro organisms used: Pure cultures of *Staphylococus*, *Streptomyces*, *E. coli*, *Bacillus* for antibacterial studies and pure culture of *Candida, A. niger, T. ressi, Penicillum* for antifungal studies; obtained from S.M.S. Medical College, Jaipur, India were used as indicator organisms. Each culture was further maintained on the same medium after every 48 h of transferring. A fresh suspension of test organism in saline solution was prepared from a freshly grown agar slant before every antimicrobial assay.

Determination of Antibacterial Assay: *In vitro* antibacterial activity of the nickel complex was studied against gram positive and gram-negative bacterial strains by the agar well diffusion method [20]. Mueller Hinton agar no.2 (Hi Media, India) was used as the bacteriological medium. The extracts were diluted in 100% Dimethylsulphoxide (DMSO) at the concentrations of 5 mg/ml. The Mueller Hinton agar was melted and cooled to 48-50ºC and a standardized inoculum (1.5×108 CFU mL^{-1}, 0.5 McFarland) was then added aseptically to the molten agar and poured into sterile petri dishes to give a solid plate. Wells were prepared in the seeded agar plates. The test compound (100 µL) was introduced in the well (6 mm). The plates were incubated overnight at 37ºC. The antimicrobial spectrum of the extract was determined for the bacterial species in terms of zone sizes around each well. The diameters of zone of inhibition produced by the agent were compared with those produced by the commercial control antibiotics, streptomycin. For each bacterial strain controls were maintained where pure solvents were used instead of the extract. The control zones were subtracted from the test zones and the resulting zone diameter was measured with antibiotic zone reader to nearest mm. The experiment was performed three times to minimize the error and the mean values are presented.

Antifungal Studies: A variety of laboratory methods can be used to evaluate or screen the *in vitro* antifungal activity of an extract or a pure compound. The most known and basic methods are the disk-diffusion and agar well diffusion methods. In this paper Antifungal activity of metal complexes was

investigated by agar well diffusion method. Herein well-known procedure, agar plates are inoculated with a standardized inoculum of the test microorganism over the entire agar surface. A hole with a diameter of 6 to 8 mm is punched aseptically with a sterile cork borer, and then a volume (20–100 µL) of the test solution of complexes at desired concentration is introduced into the well.

Here the yeasts and saprophytic fungi were subcultured onto Potato dextrose agar, PDA (Merck, Germany) and respectively incubated at 37°C for 24 hours and 25°C for 2-5 days. Suspension of fungal spores were prepared in sterile PBS and adjusted to a concentration of 106 cells mL^{-1}. Dipping a sterile swab into the fungal suspension and rolled on the surface of the agar medium. The plates were dried at room temperature for 15 min. Well of 10mm in diameter and about 7mm apart were punctured in the culture media using sterile glass tube. 0.1 mL of several dilution of the fresh extracts were administered to fullness for each well. Plates were incubated at 37°C. After incubation of 24 h bioactivities were determined by measuring the diameter of inhibition zone (in mm).For this purpose, we use ketoconazole and ciprofloxacin as standard compounds.

RESULTS AND DISCUSSION

The synthesized complex is colored and solid in nature, stable at room temperature. It is insoluble in water but moderately soluble in organic solvents like methanol, ethanol, benzene and highly soluble in binary solvent mixture.

IR Spectra: The IR spectra provide valuable information regarding coordination site of the ligand attached to the metal ion [**21, 22**]. Following table shows important characteristic of IR spectra of metal (Ni) complex (Table 1).

Table 1. IR Spectra of phenothiazine nickel oleate complex

Absorption Bands	Phenothiazine Nickel Oleate Complex	
	Observation	Reason
C-H symmetric stretching	2921 cm^{-1}	It is due to methyl ($-CH_3$) group of soap segment present in complex.
C-H asymmetric stretching	2114 cm^{-1}	
C-H symmetric stretching	2852 cm^{-1}	It is due to methylene ($-CH_2$) group of soap segment present in complex.
C-H asymmetric stretching	2849 cm^{-1}	
C-H bending (twisting and wagging)	1350 to 1390 cm^{-1}	These are small peaks due to ($-CH_2$) group present in complex.
C-H rocking	1108 cm^{-1}	Due to methyl ($-CH_3$) group.
C-H rocking	725 cm^{-1}	Due to methylene ($-CH_2$) group.
COO^- ion, C-O anti-sym stretching and sym stretching	1460to 1470 cm^{-1}	It shows the presence of fatty acid group of metal soap in the complex
Skeletal bands	1600 to 1430 cm^{-1}	It represents a heteroaromatic ring system present in metal complex.
N-O stretching and bending	1490-1460 cm^{-1} and 100-1380 cm^{-1}	It indicates the presence of nitro group in aromatic system of ligand.
C-S vibrations	750 to 610 cm^{-1}	It is due to presence of Sulphur in heteroaromatic system.
Ar-N vibrations	1300 to 1340 cm^{-1}	It shows M-L bonding

The ν(Ar-N) frequency for the complexes are observed around 1300-1340 cm^{-1}which is lower than that observed in free ligand and this evidence supports the coordination of phenothiazine nitrogen. Involvement of phenothiazine nitrogen in the complexation is also supported by the presence of a new band at 458-436 cm^{-1}, assignable to ν(M-N) for Ni(II) complex. From IR spectral data, it is evident that ligand act as a monodented, bonded to metal ion (Ni) threw secondary nitrogen atom of NH. The band near 1560 cm^{-1} characteristic of N-H bending vibration to the N-H group in the free ligand is shifted to lower frequency of 1543-1539 cm^{-1} in the complex indicate that the secondary nitrogen is the coordinating site in the complex.

NMR spectral analysis: The ^{1}HNMR spectra of free ligand and corresponding complex have been compared and determine the bonding. The signal was assigned based on chemical shifts, spin-spin interaction and their effects on substitution. Following table shows important interpretation of metal complex (Table 2).

Table 2. NMR Signal of phenothiazine nickel oleate complex

Signal of Ni oleate complex with 2-bromo-7 nitrophenothiazine	Observation
7.3 δ	Indicates the presence of aromatic C-H in the complex
8.2 δ and 7.8 δ	Represents the presence of a –I group (here $–NO_2$) in the complex
3.5 δ	Shows the presence of –N-H proton in the complex

The broad peak at 3.5 δ indicates the coordination through the –N-H group of phenothiazine molecule to the metal atom (Ni) of soap segment.

Antibacterial Analysis: To investigate antibacterial predisposition of newly synthesized nickel complex we use well diffusion method and *ciprofloxacin* is used as slandered compound for reference (Table 3).

Table 3. Antibacterial effect of Ni complex

Complex (in mg mL^{-1})	Standard (in mm)	*Staphylococcus* (in mm)	*Streptomyces* (in mm)	*E.coli* (in mm)	*Bacillus* (in mm)
20	20	--	--	--	--
40	20	--	12	--	11
60	20	--	10	--	12
80	20	--	10	--	15

According to above table it is very clear that this complex has very mild effect on bacterial strains. In fact, it does not affect some strain like *Staphylococcus* and *E.coli* at all. Its highest antibacterial effect is obtained for *Bacillus* at high concentration (Figure 1).

E. coli *Staphylococcus* *Streptomyces* *Bacillus*

Antibacterial activity of Ni-complex on *E.coli*, *Staphylococcus*, *Streptomyces* and *Bacillus*.

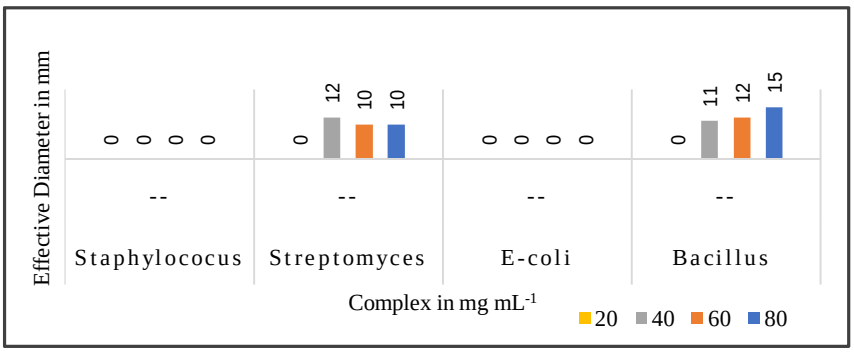


Figure 1. Bar Diagram for Antibacterial activity of Phenothiazine Ni Complex.

Antifungal Analysis: We also investigated antifungal susceptibility of Nickel oleate complex of substituted phenothiazine using well diffusion method. For this purpose, we use ketoconazole as standard compound for reference (Table 4).

Table 4. Antifungal effect of Ni complex

Complex (in mg mL^{-1})	Standard (in mm)	*Candida* (in mm)	*Aspergillus niger* (in mm)	*Trichoderma ressi* (in mm)	*Penicillium* (in mm)
20	22	08	--	--	--
40	22	--	--	10	--
60	22	--	8	08	13
80	22	--	10	13	10

Based on the results, it is suggested that well diffusion method is very useful for testing the antifungal activity of synthesized compound. It is clearly observed that the complex is very much active at different concentration (Figure 2).

T. ressi *Candida* *Penicillium* *A. niger*

Antifungal activity of Ni-complex on *T.ressi, Candida, Pencillium* and *A. niger*

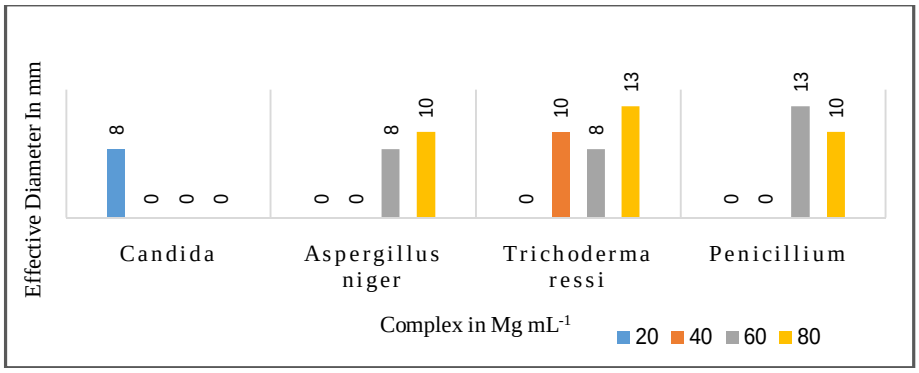


Figure 2. Bar Diagram for Antifungal activity of Phenothiazine Ni Complex.

Statistical Analysis: In this study we tried to find out effect of Ni-complex on different fungi strains and bacterial strains. For this purpose, one-way ANOVA technique was used. Hypothesis of activity of different fungi are as follows

H_0 : $\mu M_1 = \mu M_2 = \mu M_3 = \mu M_4$; that is, there is no significant different between the effects of all four fungi on the activity index of the test compound.

H_1: at least two of the mean differ, that is, there is significant different between effect of different fungi on the activity index of the test compound.

Test the above hypothesis using one-way ANOVA technique at 5% level to find out any significant difference between the activities of different fungi on test complex (Table 5). Following ANOVA table is obtained for fungal activity on complex.

Table 5. Statistical analysis by one-way ANOVA on tested fungi

Source of Variation	SS	Df	MS	F - Ratio
Between Samples	SSB = 61.5607	k-1 = 3	MSB = 20.5202	F = MSB/MSW
Within Samples	SSW = 42.1668	n – k = 7	MSW = 6.0238	= 3.4065
Total	SST = 103.7275	n -1 = 10	--	--

Here, SSB = Sum of square between, SSW = Sum of square within, SST = Sum of square total, MSB and MSW represents mean of square between and within. Here we are using $\alpha = 0.05$, so critical $F_{0.05;3,7} = 4.35$ (from F- table), while our F ratio is 3.4065. Which is less than critical value, that is, we cannot reject null hypothesis H_0. Hence, activities of different fungi on complex are significant more than 0.05.

Hypothesis of activity of different bacteria are as follows, in this study the activity was found on *Streptomyces* and *Bacillus* only, let following is the hypothesis for activity of both bacteria as follows

H_0 : $\mu M_1 = \mu M_2$; that is, there is no significant difference between the effects of both bacteria on the activity index of the test compound.

H_1: both means are different, that is, there is significant difference between effects of both bacteria on the activity index of the test compound.

Again test the above hypothesis using one-way ANOVA technique at 5% level of significance, to find out any significant difference between the activities of both bacteria on test complex (Table 6). Following ANOVA table is obtained for antibacterial activity of complex:

Table 6. Statistical analysis by one-way ANOVA on tested Bacteria

Source of Variation	SS	Df	MS	F - Ratio
Between Samples	SSB = 6.0	k-1 = 1	MSB = 6	F = MSB/MSW
Within Samples	SSW = 11.3336	n – k = 4	MSW = 2.8334	= 2.1176
Total	SST = 17.3336	n -1 = 5	--	--

Here, SSB = Sum of square between, SSW = Sum of square within, SST = Sum of square total, MSB and MSW represents mean of square between and within. Here we are using $\alpha = 0.05$, hence, from F table critical $F_{0.05;1,4} = 7.7086$, which is greater than the calculated F ratio. So null hypothesis will be correct, that is, activity index for both bacteria is significant to each other.

CONCLUSIONS

Diffusion method and results are stated in millimeter. From table 3, it has been found that this complex shows mild effect on bacterial strains of *Streptomyces* even at higher concentration, it gives good result on *Bacillus* strain and affectability increases with concentration. But this complex does not show any activity against *Staphylococcus* and *E.coli*. This indicates that nickel complex of 2-bromo-7-nitrophinothiazine give antibacterial activity to some selective bacterial strains and not a highly effective compound for this cause. The overall effect can be represented in following plot. On the other hand, pure surfactant and ligands show less inhibition whereas on complexation the inhibition enhanced and we can suggest that phenothiazine ligands are able to enhance the inhibition in the complexes.

The antifungal activities of nickel complex of 2-bromo-7-nitrophinothiazine have been evaluated by well diffusion test. The results are expressed in millimeter. It has been clear that Nickel complex shows less prominent antifungal effect on *Candida* fungus, in fact at higher concentration it does not affect *Candida* fungus (Table 4). It shows highest effect for *Trichoderma ressi* fungus at 80 mg mL^{-1}

and for *Penicillium* fungi at 60 mg mL. Results shows that mostly complex is more effective at higher concentration.

In the present investigation, it was found that complex is more active for mesophilic and filamentous fungus like *Trichoderma ressi* and soil fungus like *Penicillium*, at higher concentrations. This complex is also active for some bacterial strains like *Streptomyces* and *Bacillus* at higher concentration.

ACKNOWLEDGMENT

The authors are thankful to the principal and Head of the Department of Chemistry, Govt. P.N.K.S. College, Dausa, for providing laboratory facilities and MNIT, Jaipur for providing analytical data. Also, thanks to Seminal Lab for providing us facilities to examine biological studies.

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