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Antifungal Potential of Transition Metal Hexacyanoferrates against Fungal Diseases of Mushroom

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ABSTRACT

Ferrocyanides of Co(II), Ni(II), Cu(II), Zn(II) and Cd(II) were synthesized and characterized by IR spectra, magnetic susceptibility, thermal gravimetric analysis, elemental analysis and X ray diffraction studies. Antimicrobial potential of these complexes have been evaluated. Antifungal screening of these complexes has been carried out against *Mycogone perniciosa* and *Verticillium fungicola* causing wet and dry bubble diseases of button mushroom respectively. Nickel ferrocyanide has been found to be most effective against *Mycogone perniciosa* with 60% inhibitory effect while cadmium ferrocyanide has exhibited significant potential of 85% against *Verticillium fungicola*.

Keywords: *Verticilium Fungicola*; *Mycogone Perniciosa*; Biocidal Potential; Transition Metal Hexacyanoferrates

1. Introduction

Many of the transition metal ions in the living systems work as enzymes or carriers in macrocyclic ligand field environment. Therefore meaningful research in this direction might generate simple models for biologically occurring metallo enzymes and thus will help in developing our understanding of biological systems. These ligands are also of theoretical interest as they are capable of furnishing an environment of controlled geometry and ligand field strength [1-5]. Synthesis of a number of polydentatemacrocyclic ligands and their metal complexes has been reported in literature [6].Transition metals have an important place within medicinal biochemistry. Review of literature has revealed significant progress in utilization of transition metal complexes as drugs to treat several human diseases like carcinomas, lymphomas, infection control etc. These complexes act as therapeutic and antimicrobial agents [7-13]. Transition metals exhibit different oxidation states and can interact with a number of negatively charged molecules. This activity of transition metals has started the development of metal based drugs with promising pharmacological application and may offer unique therapeutic opportunities. To provide an update on recent advances in the medicinal use of transition metals, a Medline search has been carried out to identify the recent relevant literature [14,15].

These complexes may possess antimicrobial activity against pathogenic fungi being used as a test organism in the present study. It is well established that metal ferrocyanides acts as adsorbent[16,17], ion-exchangers [18, 19] and photosensitizers [20].Transition metals such as zinc, copper, cobalt, manganese, iron have been reported to be essential for crops. They remain in soil in small quantity and known as micronutrient. If the deficiency of these elements is detected in soil these are recommended to be added to soil with fertilizer or in form of top dressing. Thus these metals act as micronutrient in trace quantity and hence application of metal complexes in combination with other ecofriendly chemicals/botanicals may be evaluated for antimicrobial potential.

Mushrooms can provide more than just taste and texture for our meals-they actually have a surprisingly high nutritional value also. White button mushrooms have a surprising amount of nutrients including niacin, riboflavin, folate, phosphorus, iron, panthothenic acid, zinc, potassium, copper, magnesium, vitamin B6, selenium and thiamin. In addition, white button mushroom extract has been found to reduce the size of some cancer tumors and slow down the production of some cancer cells. It is most prominently linked to reducing the risk of breast and prostate cancer. The yield of the crop is severely affected by fungal pathogens *Mycogone perniciosa* and *Verticillium fungicola* causing wet and dry bubble diseases of button mushroom respectively. During the last decade V. fungicola has become less sensitive to the only approved chemical (prochloraz) that is still effective to treat infection. Moreover, it is expected that prochloraz will be banned from commercial mushroom growing. Therefore, alternative strategies to control the disease are urgently needed. Wet bubble caused by *M. perniciosa* is a disease that often occurs on mushroom farms. It can be of very severe (when there are practically no healthy mushrooms left on the beds), and not that much (unitary diseased mushrooms) depending on the time when the infection occurred; and the degree of infection.

Keeping in view the above facts present study has been undertaken to synthesize, characterize and evaluate antifungal activity of complexes of Mn(II), Co(II), Ni(II), Cu(II), Zn(II) and Cd (II) against *Mycogone perniciosa* and *Verticillium fungicola* causing wet and dry bubble diseases of mushroom respectively.

2. Materials and Methods

2.1. Synthesis of Metal Ferrocyanides

Six transition metal ferrocyanides namely manganese ferrocyanide, cobalt ferrocyanide, nickel ferrocyanide, copper ferrocyanide, zinc ferrocyanide and cadmium ferrocyanide were synthesized following Kourim's method [21]. A solution of potassium ferrocyanide (167 ml, 0.1 M) was added to solution of desired metal salt (500 ml, 0.1 M) with constant stirring at room temperature. A slight excess of metal salt solution markedly improves the coagulation of the precipitate. The reaction mixture was heated on a water bath at 80˚C for 3 - 4 hrs, and allowed to stand at ambient temperature for 24 hrs. The precipitate was filtered under vacuum and washed thoroughly with double distilled water. It was dried in an oven at 60˚C. The dried product was ground and sieved to 100 mesh size. Coloured powders thus obtained were stable on exposure of air and moisture. All the synthesized complexes were found to be insoluble in water. These were characterized on the basis of elemental analysis, carried out on Carlo Erba 1108 CHN analyzer and Atomic Absorption Spectrophotometer (Perkin Elmer 3100), IR spectra (recorded on Bio-Rad FTIR spectrophotometer), magnetic susceptibility measurement (recorded on VSM-155), molar conductivity measurement and X ray diffraction studies. The data has been reported in **Tables 1-9**.

2.2. Collection of Fungal Cultures

Two fungal pathogens namely Mycogone perniciosa and Verticillium fungicola causing wet and dry bubble diseases of button mushroom respectively, have been collected from Department of Plant Pathology, College of Agriculture, G.B. Pant University of Agriculture and Technology, Pantnagar. Both these fungal pathogens were grown on potato dextrose agar (PDA) medium and incubated at 20˚C and 28˚C respectively.

2.3. Screening of Metal Complexes for Fungicidal Activity

Paper disc method, based on diffusion capacity of test chemical(s) through agar medium has been used for preliminary screening of antifungal activity of metal complexes [22]. Fungal plug were placed at the center of assay plate containing sterilized PDA and allowed to grow. After circular growth of about 2 - 3 cm diameter four sterilized paper disc (two loaded with 20 μl aqueous sus-

Table 1. Elemental analysis data of metal ferrocyanides.

Metal Complexes	% found (calculated)				
	Metal	Fe	N	C	H
MnFC	28.56 (29.23)	14.66 (14.86)	22.59 (22.36)	20.67 (19.17)	1.69 (1.61)
CoFC	32.12 (32.22)	15.30 (15.27)	21.16 (22.97)	19.65 (19.70)	1.11 (1.10)
NiFC	27.85 (27.93)	13.00 (13.28)	18.79 (19.19)	16.51 (17.14)	2.22 (2.30)
CuFC	27.10 (27.32)	12.10 (12.01)	18.12 (18.07)	14.75 (15.49)	3.13 (3.03)
ZnFC	32.84 (32.95)	14.10 (14.08)	20.40 (21.18)	17.74 (18.16)	1.51 (1.45)
CdFC	50.12 (51.47)	12.58 (12.79)	20.38 (19.24)	17.71 (16.50)	0.26 (0.00)

Table 2. Infrared spectral peak assignment of metal ferrocyanide complexes.

Complexes	Adsorption frequencies (cm^{-1})				
	ν_{HOH}	$\nu_{C\equiv N}$	HOH bending	ν_{Fe-C}	$\nu_{Metal-N}$
$Mn_2[Fe(CN)_6]\cdot 3H_2O$	3701	2070	1631	592	451
$Co_2[Fe(CN)_6]\cdot 2H_2O$	3724	2083	1609	592	465
$Ni_2[Fe(CN)_6]\cdot 5H_2O$	3697	2091	1611	592	463
$Cu_2[Fe(CN)_6]\cdot 7H_2O$	3845	2090	1621	592	503
$Zn_2[Fe(CN)_6]\cdot 3H_2O$	3685	2080	1600	603	496
$Cd_2[Fe(CN)_6]$	3724	2071	1623	590	508

Table 3. Magnetic moments and molar conductivity of metal ferrocyanide complexes.

Metal hexacyanoferrate (II)	μ_{calc} (B.M.)	μ_{eff} (B.M.)	Molar conductance (μS)
$Mn_2[Fe(CN)_6]\cdot 3H_2O$	5.92	6.21	24.2
$Co_2[Fe(CN)_6]\cdot 2H_2O$	3.87	4.36	9.81
$Ni_2[Fe(CN)_6]\cdot 5H_2O$	2.83	2.99	6.61
$Cu_2[Fe(CN)_6]\cdot 7H_2O$	1.73	2.45	6.72
$Zn_2[Fe(CN)_6]\cdot 3H_2O$	0.00	0.81	2.70
$Cd_2[Fe(CN)_6]$	0.00	0.90	7.44

Table 4. Major X-ray absorption peaks in the XRD spectra of manganese ferrocyanide.

2θ	d-Spacing(Å) observed	Relative intensity (%)	d-Spacing[Å] reported in PCPDF database
17.6155	5.0348	56.48	5.8087
24.9795	3.56478	100.00	3.5570
29.6726	3.0117	7.07	3.0334
39.1584	2.3005	6.69	2.3081
40.0277	2.2525	9.91	2.5152
43.4091	2.0846	5.28	2.9043

Table 5. Major X-ray absorption peaks in the XRD spectra of cobalt ferrocyanide.

2 θ	d-Spacing(Å) observed	Relative Intencity (%)	d-Spacing[Å] reported in PCPDF database
17.7134	5.0072	60.23	5.0300
25.0657	3.5527	100.00	3.5600
35.8547	2.5045	64.74	2.5300
43.7255	2.0702	8.97	2.0800
44.9538	2.0165	13.02	2.2800

Table 6. Major X-ray absorption peaks in the XRD spectra of nickel ferrocyanide.

2 θ	d-Spacing(Å) observed	Relative intensity (%)	d-Spacing[Å] reported in PCPDF database
17.7146	5.0069	60.93	5.0500
25.0107	3.5604	100.00	3.5700
35.7078	2.5145	53.31	2.5700
40.1426	2.2463	10.26	2.2600
44.0851	2.0542	15.62	2.0600
51.3617	1.7789	10.64	1.7840
54.7539	1.6765	4.39	1.6830
57.9877	1.5891	11.22	1.5230

Table 7. Major X-ray absorption peaks in the XRD spectra of copper ferrocyanide.

2 θ	d-Spacing(Å) observed	Relative Intencity (%)	d-Spacing[Å] reported in PCPDF database
25.1752	3.5375	79.69	3.5000
29.7271	3.0054	7.41	3.0200
36.0522	2.4913	36.50	2.5000
40.3144	2.2372	14.79	2.2300
44.3532	2.0424	12.34	2.0400

Table 8. Major X-ray absorption peaks in the XRD spectra of Zinc ferrocyanide.

2 θ	d-Spacing(Å) observed	Relative Intencity (%)	d-Spacing[Å] reported in PCPDF database
16.3677	5.4157	100.00	5.4000
19.7227	4.5014	46.65	4.5100
21.7924	4.0783	90.70	4.0800
28.6684	3.1139	22.18	3.1100
29.7535	3.0027	9.27	3.0000
35.6073	2.5141	10.35	2.5400
37.7830	2.3810	7.67	2.3700
38.8405	2.3186	7.21	2.3200
40.9696	2.2029	11.16	2.2000
47.8545	1.9008	5.80	1.9500

Table 9. Major X-ray absorption peaks in the XRD spectra of cadmium ferrocyanide.

2 θ	d-Spacing(Å) observed	Relative intensity (%)	d-Spacing[Å] reported in PCPDF database
19.5467	4.5415	3.45	4.1100
28.7088	3.1096	19.86	3.1600
31.7556	2.8178	3.52	2.8300
35.3196	2.5412	39.74	2.4900
39.6586	2.2726	19.20	2.2300
42.7467	2.1153	9.50	2.1100
49.1373	1.8541	1.50	1.8180
50.7909	1.7976	7.34	1.7470
57.3244	1.6073	10.31	1.6670
59.3363	1.5575	2.77	1.5760
61.4229	1.5095	1.43	1.5350
66.3195	1.4094	2.20	1.4740

pension of metal ferrocyanides and two with sameamount of distilled water) were placed at equal distance from center in order to see the effect of metal ferrocyanides on the growth of fungal pathogen. Inhibition zones were measured after 36 hrs of incubation. Dumb bell shaped growth of fungus was observed in case of metal complex possesing growth inhibitory component(s).

Food poisoning technique was used to find percent inhibition. For this purpose 0.375% (w/v) metal complex was spread to each petri-dish containing the sterilized media, while in control treatment equal amount of pure solvent was added. The fungal plug was placed at the centre of petri-dish. Growth of fungus was recorded after 72 hrs of incubation. The percent inhibition was calculated using the formula of Vincent [23].

Percent Inhibition = (C-T)/C × 100

Where C is the growth in control in mm and T is growth in treatment in mm.

All the experiments were carried out in triplicate in randomized block design and average value was used for interpretation of results (**Tables 5-9**).

2.3.1. Correlation Coefficient (*r*)

The correlation coefficient (r) was calculated using the following equation,

$$r = \frac{n\sum xy - \sum x \sum y}{\sqrt{\left\{\left[n\sum x^2 - \left(\sum x\right)^2\right]\left[n\sum y^2 - \left(\sum y\right)^2\right]\right\}}}$$

Here n is the number of data points.

1) $r = +1$, perfect positive correlation, increase in one variable is accompanied by the increase in the other.

2) $r = -1$, perfect negative correlation, decrease in one variable is accompanied by the decrease in the other.

2.3.2. Coefficient of Determination ("r^2")

Although correlation coefficient is a good measure of the strength of the association, but it has got no literal interpretation. The squared values of r, r^2 called coefficient of determination, however have a very clear meaning. It gives the measure of the proportion of variation in one variable associated with variations in the other. For example, if the value of $r = 0.8$, then $r^2 = 0.64$. It means that 64% variations in the value of inhibition zones are associated with variation in metal complex and the remaining 36% can be attributed to some other unknown factors. The value of r^2 ranges from 0 to 1.

2.3.3. Significance Test

The significance test (t test) was performed and values of was calculated using the formula:

$$t = r\frac{\sqrt{(n-2)}}{\sqrt{1-r^2}}$$

Here, n is number of observations.

The observed value of "t" is compared with the critical value of t obtained for n-2 degrees of freedom at 5% significance level from the t distribution table [24].

3. Results and Discussion

3.1. Characterization of Metal Ferrocyanides

The molecular formula of synthesized metal complexes has been established on the basis of elemental analysis (**Table 1**) and thermal studies. Assignments of infra red peaks have been reported in **Table 2**. A broad band in the range of 3400 - 3750 cm^{-1} has been observed due to interstitial water molecules and OH^- groups while the characteristic HOH bending appears at 1600 - 1631 cm^{-1} in case of all the complexes synthesized. A sharp peak at 2080 ± 10 cm^{-1} is characteristic of cyanide stretching. Sharp peaks at 691 - 590 cm^{-1} are characteristic of Fe-C stretching frequencies. $\nu_{Metal\text{-}Nitrogen}$ was observed at 451 - 508 cm^{-1}.

Values of observed and calculated magnetic moments have been reported in **Table 3**. From a structural stand point, the ferrocyanide ion can be considered to be a good example of strong field (low spin) octahedral complexes. In the presence of the strongly perturbing cyanide ligand the 3d orbitals of ferrous ion will get splitted, causing a relatively large separation between t_{2g} and e_g orbitals. In the ground state, therefore, the six electrons from Fe (II) ion will be placed in the low lying t_{2g} orbitals. The metal ions like Zn^{2+}, Co^{2+}, Cu^{2+}, Cr^{3+}, Ni^{2+}, Mn^{2+} and Cd^{3+} will remain in the lattice.

All synthesized metal ferrocyanides are expected to be diamagnetic due to paired electrons. However, the outer cations may contribute to the observed magnetic moment, if any. The magnetic moment of Mn, Cu, Co and other ferrocyanides and found that zinc and cadmium ferrocyanides are diamagnetic as expected. Observed magnetic moment values (**Table 3**) of these metal hexacyanoferrates were found to be in good agreement with calculated values. μ_{obs} indicate presence of three unpaired electrons in cobalt ferrocyanide, which is in agreement with d^7 configuration of Co^{2+}, whereas reported value of magnetic moment for cobalt ferrocyanide is 4.6 BM. μ_{obs} values revealed that five, three, two and one unpaired electrons are present in Mn(II), Co(II), Ni(II), and Cu(II) hexacyanoferrates respectively, while zinc and cadmium hexacyanoferrates have zero magnetic moments.

Conductivity measurements (**Table 3**) in non aqueous solutions provide a method for testing the degree of ionization of the complexes. The value of molar conductance the soluble complexes in DMSO indicate these complexes to be poor electrolytes.

TG and DTA studies were carried out to confirm the presence of lattice water in metal hexacyanoferrates. Mass loss was found to be equivalent to three, two, five, seven, and three moles of water in case of Mn(II), Co(II), Ni(II), Cu(II), and Zn(II) hexacyanoferrate respectively. Cadmium ferrocyanide did not show any loss of water molecule. Molecular formula determined on the basis of elemental analysis, TG and DTA are as follows:

$Mn_2[Fe(CN)_6]\cdot 3H_2O$, $Co_2[Fe(CN)_6]\cdot 2H_2O$,
$Ni_2[Fe(CN)_6]\cdot 5H_2O$, $Cu_2[Fe(CN)_6]\cdot 7H_2O$,
$Zn_2[Fe(CN)_6]\cdot 3H_2O$, and $Cd_2[Fe(CN)_6]$.

The synthesized metal complexes were characterized by X ray diffraction studies (**Figure 1-6**). d values of the observed peaks have been reported in **Table (5-9)** which are in good agreement with the published data for manganese, cobalt, nickel, copper, zinc and cadmium ferrocyanides in PC-PDF file numbers 46-0910, 23-0188, 14-0291, 01-0244, 24-0164, and 01-0433 respectively.

3.2. Antifungal Potential

Antifungal potential of metal complexes against *M. perniciosa* and *V. fungicola* has been reported in **Table 10**. Manganese, nickel, copper and zinc ferrocyanides have exhibited inhibition zones in the range of 2 - 4 mm with percent inhibition ranging 30% - 60% against *M. pernici osa*. Nickel ferrocyanide possesses maximum inhibitory-effect against wet bubble causing pathogen *M. perniciosa* showing 60% growth inhibition.

Manganese, cobalt, nickel, copper and cadmium ferrocyanide have exhibited inhibition zone ranging 2 - 18 mm and percent inhibition in the range of 4% - 85% against *V. fungicola.* Cadmium ferrocyanide has been found to be most effective against *V. fungicola* showing 85% growth inhibition. All the metal ferrocyanides except zinc ferrocyanide exhibit significant activity against *V. fungicola*.

The values of correlation coefficient ("r") and coeffi-

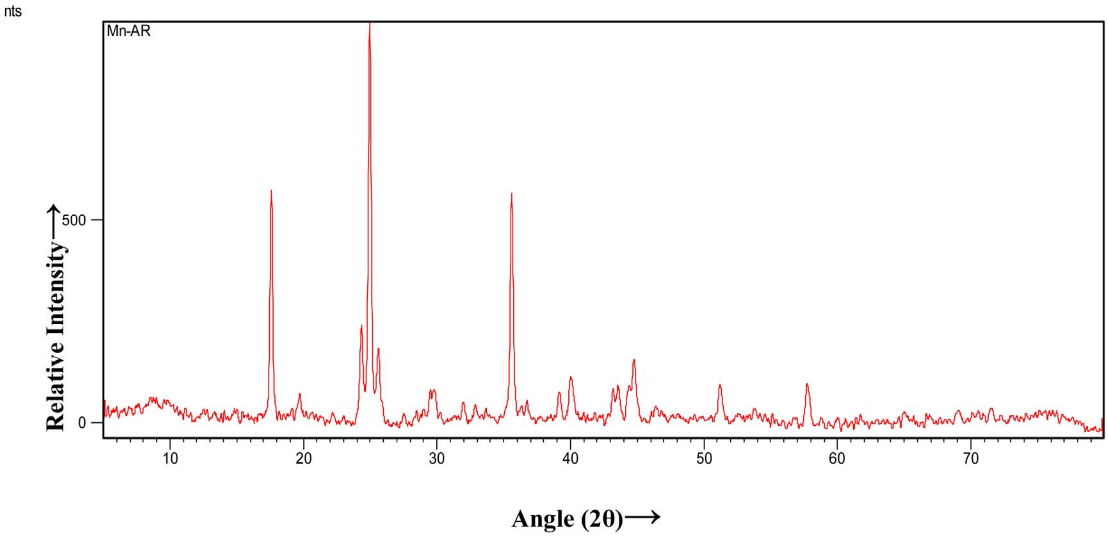

Figure 1. X-ray diffraction pattern for manganese ferrocyanide.

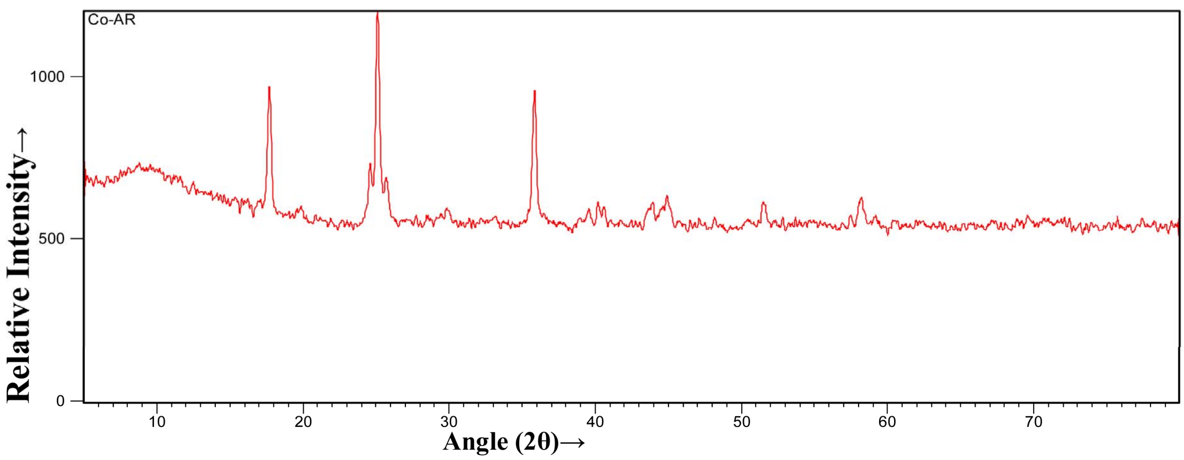

Figure 2. X-ray diffraction pattern for cobaltferrocyanide.

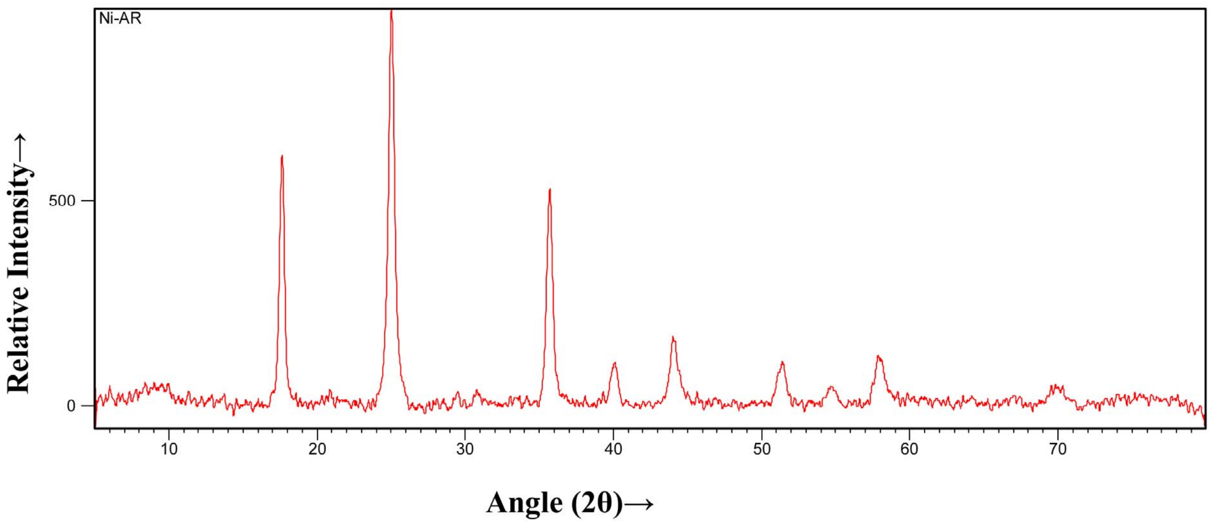

Figure 3. X-ray diffraction pattern for nickel ferrocyanide.

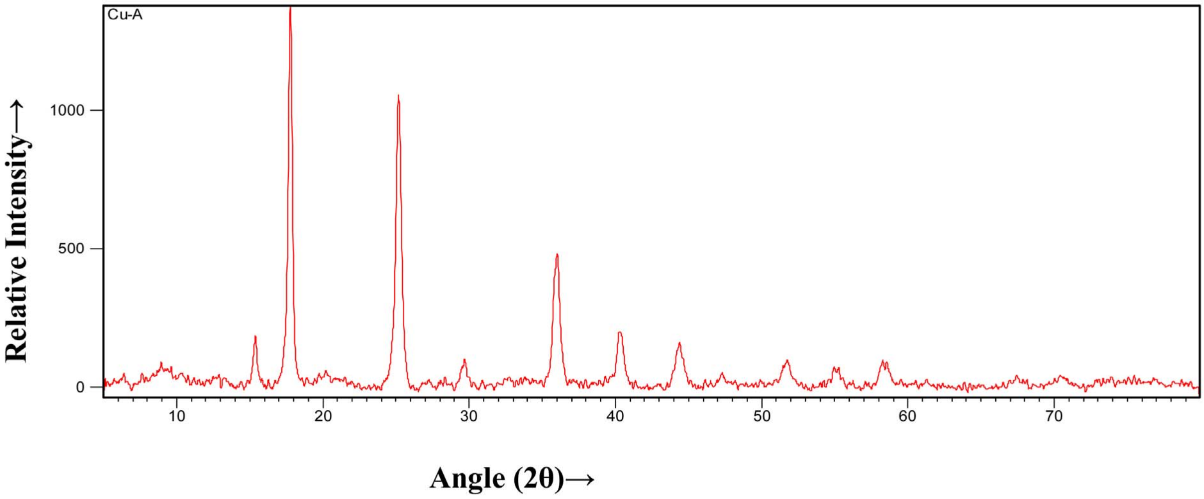

Figure 4. X-ray diffraction pattern for copper ferrocyanide.

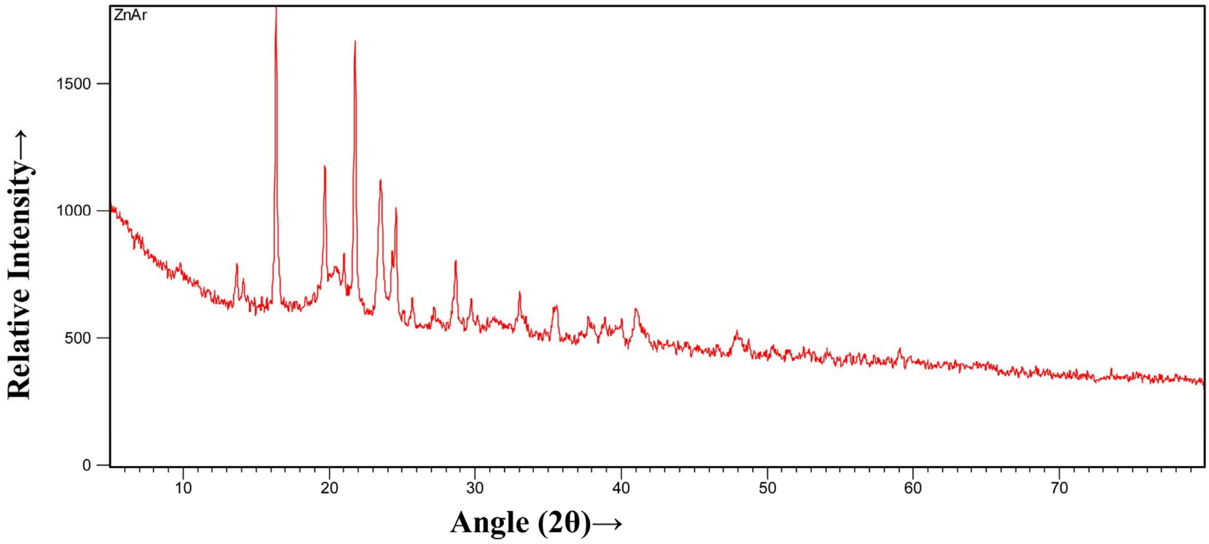

Figure 5. X-ray diffraction pattern for zinc ferrocyanide.

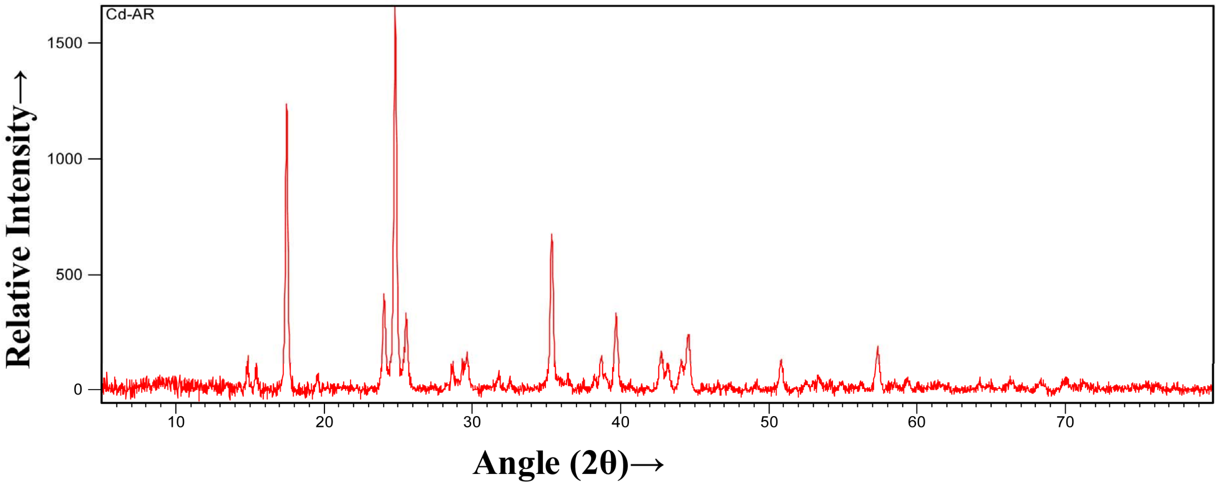

Figure 6. X-ray diffraction pattern for cadmium ferrocyanide.

Table 10. Antifungal activity of transition metal ferrocyanides.

Metal ferrocyanides	Fungal pathogen *Mycogoneperniciosa*		Fungal pathogen *Verticelliumfungicola*	
	Inhibition zone (mm)	Percent inhibition	Inhibition zone (mm)	Percent inhibition
$Mn_2[Fe(CN)_6]\cdot 3H_2O$	2	30	7	32
$Co_2[Fe(CN)_6]\cdot 2H_2O$	0	00	8	38
$Ni_2[Fe(CN)_6]\cdot 5H_2O$	4	60	2	5
$Cu_2[Fe(CN)_6]\cdot 7H_2O$	3	50	15	75
$Zn_2[Fe(CN)_6]\cdot 3H_2O$	2	30	-	-
$Cd_2[Fe(CN)_6]$	0	00	17	85

The values of correlation coefficient ("r") and coefficient of determination ("r^2") are 0.997 and 0.994 respectively for observations related to the inhibitory effect against *M. perniciosa*. The value of "r^2" suggests that 99.4% inhibition was caused by metal ferrocyanides and rest 0.6% may be attributed to other unknown and uncontrolled factors. The calculations related to the significance test ("t" test) revealed that the value of "t" (25.73) is much higher than the critical value noted from "t" distribution table for degree of freedom 4 at 5% significance level. This suggests that there are less than 5% chances of error in drawing the conclusions.

The calculated value of "r", "r^2", and "t" (at 5% significance level), for the observations made in case of *V. fungicola* are 0.999, 0.998 and 51.50 respectively. The value of "t" is much higher than the critical value which is indicative of less than 5% chances of occurrence of error, and that the null hypothesis may be safely rejected at 5% significance level.

There are few reports on synergistic effect of antimicrobial activity of metal ferrocyanide with botanicals [13]. These complexes have also been reported to adsorb biomolecules. Hence these may be proved to be potential solid support for plant based biocidal component(s). There may be the possibility of adsorption of active ingredient(s) at the surface of transitional metal ferrocyanides. Thus concentration, efficiency and shelf life of active chemical(s) may increase and lead to increased activity (biopotentiation). These studies will be helpful in development of new fungicidal formulations for management of dry and wet bubble diseases of button mushroom.

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