
Adsorptive Interactions of Natural Antifungals with Metal Ferrocyanides and its Pharmaceutical Potential

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ABSTRACT

The study aimed to investigate the adsorptive interactions of metal hexacyanoferrates (II) and ethanol and acetone extracts of natural antifungals. Nickel, manganese and copper hexacyanoferrates (II) were synthesized and characterized by elemental and spectral studies. Each metal hexacyanoferrate (II) was combined with two natural antifungals: coconut (*Cocos nucifera*) and ochro (*Abelmoschus esculentus*) leaf extract. The antifungal potential was executed by the disc diffusion method. The acetone and ethanol extract of *A. esculentus* at 10 mg/m L paired with copper and nickel ferrocyanides, respectively were found to possess the most effective antifungal potential. Each interaction pair was further analyzed through infrared spectral studies and phytochemical analysis. The interaction of natural antifungals *Azadirachta indica* (neem), *Ocimum sanctum* (tulsi), *Cassia obtusifolia* (money bush), *Cassia alata* (canicra bush) and *Tagetes patula* (marigold) with manganese, silver and titanium ferrocyanides and their medical applications were also evaluated.

Keywords: Cocos nucifera; Abelmoschus esculentus; Natural antifungals; Adsorptive interaction; Metal ferrocyanide; Candida albicans; Disc diffusion; Pharmaceutical potential.

1. INTRODUCTION

Metal hexacyanoferrates have interesting solid-state chemistry and structural attributes, these compounds have garnered intense recent interest because of their electrocatalytic, electrochromic, ion-exchange, ion-sensing, and photo-magnetic properties [1]. Metal hexacyanoferrates (II) serve three functions: as adsorbents, ion exchangers and photosensitizers [2]. Biosorption of heavy metals has been a recent research area where plants, bacteria and fungi facilitate the degradation of metal-cyanide complexes, including large complexes such as ferricyanides and ferrocyanides. The biodegradation occurs when the impermeable membrane of the metal-cyanide complexes is disrupted [3]. Biosorption is one of the significant properties of both living and dead microorganisms (and their components) relevant for treatment of pollutants [4, 5].

In other cases, the fungi do not tolerate the toxicity of the heavy metal and its complexes at high concentrations. Metals and their compounds can interact with fungi in various ways depending on the type of metal, organism and environment [6]. They exert toxic effects in many ways, for example by blocking the functional groups of enzymes [7]. Some of the mechanisms by which metal hexacyanoferrates (II) function to degrade the fungi instead are: inhibiting cell division, enzyme activity, disrupting cell membrane and denaturing of protein among other actions [8,9]. It can be assumed that either the action of the fungi to degrade the metal-cyanide complex would result in its decrease in growth or the fungi would be intolerable to the toxicity of the metal hexacyanoferrates (II). Filamentous fungi were reported to exhibit considerable tolerance towards heavy metals and become dominant organisms in some polluted habitats [10]. The effects of interacting natural antifungals with transition metal ferrocyanides. The transition (II) metal ferrocyanides included nickel, copper, manganese which interacted with methanolic leaf extracts. The effects were observed using scanning

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electron microscope. The interaction was tested in against *Rhizoctonia solani*, a fungal disease which affects potatoes. Virulent *R. solani* forms infection cushions on the surfaces of host sprouts and roots, and penetration by the pathogen occurs only under these areas [11]. One of the leaves extracts in particular *Cassia alata* (Canicro bush) was least effective when paired with nickel ferrocyanide. The effects of the leaves extracts paired with manganese ferrocyanide was greater, while the leaf extracts paired with copper ferrocyanide was the greatest [12].

The experiment was repeated with the same metal ferrocyanides and fungal disease. The only difference being the methanolic leaf extracts. The results remained unchanged with cadmium ferrocyanide being the most effective followed by manganese and nickel ferrocyanides [13]. The antifungal activity of nickel and copper ferrocyanide were tested against *Aspergillus niger* and *Candida albicans*. Neither of the ferrocyanides showed any significant effectiveness against *C. albicans*[14]. Metal ferrocyanides: nickel, copper and manganese against *Verticillium fungicola* and *Mycogone perniciosa*. *M. perniciosa* (wet bubble disease) and *V. fungicola* (dry bubble disease) are fungal diseases which affect mushrooms. All of the ferrocyanides exhibited antifungal potential against *M. perniciosa*. Synthesis of a number of poly- dentatemacrocyclic ligands and their metal complexes has been reported in literature [15]. The main findings revealed that nickel ferrocyanide was most effective in inhibiting the activity of *M. perniciosa* [16]. The antifungal potential of nickel and copper ferrocyanides was tested against the dry bubble disease (*V. fungicola*) of *Agaricus bisporus*. The results concluded that both ferrocyanides has some amount of effectiveness against the fungal disease [17].

The *C. nucifera* oil contains medium fatty acids such as caprylic acid and lauric acid. These fatty acids are the antifungal agents [18]. Recently, the antifungal activity of several lauric acid derivatives such as D-laurate A, T-laurate A, Span-20 and Tween 20, monolaurylglycerol and sucrose esters were demonstrated to inhibit mould growth [19]. The oil has been linked to fight against fungal infections such as ringworm, Athlete's foot, toenail fungus [20] and dandruff [21]. *C. nucifera* oil cream was created using varying amounts of *C. nucifera* oil (sourced from coconut fruit). The creams were tested on skin inoculated with *Candida albicans*, *Aspergillus niger*. After the cream was applied, five (5) minutes later the area was swabbed and then incubated overnight. The coconut oil creams exhibited antifungal potential which was boosted due to the presence of an emulsifying agent which allowed monolaurin to acquire an increased penetration of the skin. Inhibition of the growth of *Aspergillus niger*, *Candida albicans* began before the first hour was achieved and was maintained for 1-3 weeks [22]. Kabara et al. [23] demonstrated that lauric acid, oleic acid, and linoleic acid possessed biocidal effects against gram-positive bacteria. This study is well thought out because of the objectives to formulate creams which not only fight fungi with the use of *C. nucifera* oil but also consider its stability, that is, how it would interact with the additional components, and with human skin which has been infected. Further research and testing done on the successful batches of the formulation could lead to an alternative treatment. Previous research on plant oils have demonstrated that almond, jojoba, soybean, and avocado oils, when applied topically, mostly remain at the surface of skin, without deep penetration into the first upper layers of the stratum corneum [24].

In 2016 a research was published on the disease of early childhood caries (ECC), which occurs when a child's teeth are decayed, missing or their surfaces are filled. ECC is caused by microorganisms such as *C. albicans* which affects children who are 6 years 11 months or younger. The fungus was isolated in children affected with ECC, its susceptibility was tested against 0.2% chlorhexidine, *C. nucifera* oil obtained from the fruit, probiotics and 2% ketoconazole. All of the antifungal agents were found to inhibit the growth of the fungus. However, chlorhexidine and coconut oil demonstrated the highest antifungal activity which can be compared to ketoconazole [25]. Kim et al. [26] demonstrated that coconut oil increased expression of CE components, thereby contributing to protective barrier functions of the stratum corneum. An investigation of the antifungal activity of ethanol, chloroform, acetone, petroleum ether and aqueous extracts of coconut shell against *Epidermophyton*, *A. niger*, *Penicillium*, *Microsporum*, *C. albicans* and *A. flavus*. All of the extracts demonstrated an increase in the antifungal effectiveness as the concentration increased [27].

Another constituent of coconut which has antifungal potential is the milk kefir. The coconut milk kefir was tested against *S. cerevisiae* and *A. niger*. The most effective treatment was at 175µl and 200µl

against *S. cerevisiae* and *A. niger* respectively [28]. Coconut milk and kefir have each been documented to have health benefits. The aqueous extract of coconut is found to possess antineoplastic, antifungal and antimicrobial effects [29].

Green coconut water has also been tested against *C. albicans*. At increasing concentrations 250µg, 500µg and 1000µg, the potential increased. At 1000µg the zone of inhibition was 14 ± 0.3 mm when compared to amphotericin B's result of 15 ± 0.3 mm [30].

Abelmoschus esculentus / *Hibiscus esculentus* contains flavones and triterpenoids which are the antifungal agents that fight against *A. flavus*, *A. niger* and *C. albicans* [31,32]. The antifungal potential of *A. esculentus* involved the synthesis of gold nanoparticles using seed aqueous extract obtained from plant and tested on fungal diseases, *C. albicans* being one. The antifungal potential of gold nanoparticles containing the seed aqueous extracts was evaluated by the maximum zone of inhibition of each concentration which was compared to amphotericin B (positive control). Gold nanoparticles (AuNPs) are a promising class of nanomaterials with many varieties of applications, which includes cancer hyperthermia treatment [33], surface-enhanced Raman spectroscopy (SERS) [34], and infrared radiation absorbing optics [35].

The antifungal potential of the nanoparticles was greater than that of amphotericin B at higher volumes against the fungi. This advance research reveals the pairing of the natural antifungal potential of seed aqueous extract of *A. esculentus* and advancement in nanotechnology, which enhanced the antifungal ability of the plant's seeds [36]. The entire *A. esculentus* plant was and tested against three fungi: *C. albicans*, *A. niger* and *S. cerevaceae*. The crude methanolic extracts and ciprofloxacin were applied to each microorganism where the growth of *A. niger* was the most inhibited; followed by *C. albicans* ciprofloxacin demonstrated greater inhibitions than the extracts where *C. albicans* was the most susceptible [37]. Bhalodia & Shukla [38] experimented for the zone of inhibition of extracts, and the results were compared with that of different standards like ampicillin, ciprofloxacin, norfloxacin, and chloramphenicol for antibacterial activity and nystatin and griseofulvin for antifungal activity. The results showed that the remarkable inhibition of the bacterial growth was shown against the tested organisms. Gandra et al. [39] has reported antifungal potential of copper (II), manganese (II) and silver (I) 1, 10 – phenanthroline chelates against multi drug – resistance fungal species forming the candida haemulonii complex. A review on pharmacological applications of vanadium (IV), vanadium (II), chromium (III), manganese (II), iron (II) and cobalt (II) with Schiff base complexes was described by Hossain et al. [40]. The ethyl acetate extract of the flower of *A.esculentus* was tested against *C. albicans* and compared to fluconazole. At concentrations 40 mg/ml and 50 mg/ml the extract produced a zone of inhibition of 16 mm and 22 mm, respectively when compared to fluconazole's value of 18 mm [41]. Recently, fluconazole-resistant *C. albicans* strains and intrinsically resistant *Candida* species, such as *C. glabrata* and *C. krusei*, have emerged in immunocompromised patients receiving therapy or prophylactic treatment [38, 42,].

A search of literature indicated that very few reports are available from our laboratory on interaction of natural antifungals with metal hexacyanoferrates (II) and their medical applications [43, 44].

In view of this, an attempt was made to study the phytochemical analysis and the antifungal potential of some natural antifungals and metal hexacyanoferrates (II). In addition, the present work describes phytochemical analysis and antifungal potential of natural antifungals: *C. nucifera* and *A. esculentus* leaves extract (ethanol and acetone) and metal hexacyanoferrates (II).

2. METHODOLOGY

2.1 Chemicals

Potassium ferrocyanide, Nickel (II) chloride, Manganese (II) chloride, Copper (II) chloride was obtained from BDH, Poole, England. Nystatin, Picric acid, ferric chloride, metal magnesium and acetic anhydride were obtained from Aldrich. All chemicals were of analytical reagent grade and used without further purification. Solutions were prepared in doubly distilled water.

Microorganism: Candida albicans and *Media: Mueller Hinton*, BD BBL was obtained from Department of Biology, University of Guyana.

2.2 Synthesis of Metal Hexacyanoferrates (II)

The copper, manganese and nickel hexacyanoferrates (II) were synthesized by similar method reported in literature by Kourim, et al. [45]. 500 mL, 0.1 M of the metal (II) chloride solution was added to 167 mL, 0.1 M of potassium ferrocyanide solution with constant stirring.

The reaction mixture was heated on a water bath at 85°C for 4 hours, occasionally being stirred.

Afterwards the mixture was kept at room temperature for 24 hours. The mixture was filtered using Whatman filter paper and washed several times with distilled water. The residue was dried in an oven at 60°C to remove the moisture. The dried product was ground with a mortar and pestle and sieved to 125 µm particle size.

2.3 Characterization of Metal Hexacyanoferrates (II)

The synthesized copper, manganese and nickel hexacyanoferrates (II) were found to have dark brown, grey and light green colours, respectively. Elemental analysis: Atomic absorption spectrophotometer was used to analyze the amount of iron, metal, carbon, hydrogen and nitrogen. These values are given in Table 1. The spectral analysis of each metal hexacyanoferrates (II) was done using Infrared Spectrophotometer. The spectral analysis will be recovered in KBr disc using KBr pellets of **BUCK Scientific M530**. All of the samples were placed in the infrared spectrophotometer and read at 4000 cm⁻¹ - 400 cm⁻¹. The infrared spectral data of the metal ferrocyanides are given in Table 2. The infrared spectral peaks around 3500 cm⁻¹ and 1600 cm⁻¹ characteristics of water molecules / OH groups and HOH bending, respectively. Two sharp bands around 2000 cm⁻¹ and 580 cm⁻¹ are characteristics of cyanide and Fe - C stretching, respectively. Another sharp band at 500 cm⁻¹ probable shows the presence of metal-nitrogen band due to polymerization.

2.4 Preparation of Natural Antifungal Extracts

Extraction for all leaf samples, a modified procedure is reported by Mworio et al. [46].

The leaves were picked, washed with tap water and dried at room temperature to remove the excess water. The sample was ground using a Wiley Mill. A 50 g of the ground sample was soaked in 200 mL of acetone. The soaking sample via cold percolation was kept for 48 hours at room temperature. The acetone-sample extract was decanted and filtered every 48 hours using Whatman filter paper no. 41 ash less and fresh acetone was added. This was repeated three times. The acetone extracts were pooled and concentrated using rotary evaporator (40°C, 70-80 k Pa), until a dry crude was obtained. The residue of the sample left after the acetone extraction was allowed to dry.

The dried sample was further extracted with ethanol. The ethanol extract obtained was concentrated using rotary evaporator (50°C, 90 k Pa).

2.5 Plate Preparation

Distilled water was added to Mueller Hinton agar; with frequent agitation the mixture was heated to a slow boil until the powder was dissolved. Mueller Hinton agar was autoclaved at 259°F at 20 psi for 20 minutes. After removal from the autoclave Mueller Hinton agar was allowed to cool to room temperature for comfortable handling. The cap was removed.

The mouth of the flask containing the Mueller Hinton agar was placed over a flame for a few seconds. This was done to prevent any organism in the area from falling into the flask. The molten agar was poured slowly into the base of the sterile petri dish and covered with the lid under a laminar air flow cabinet. The petri dishes were left to solidify for approximately 30 minutes and UV sterilized for 20

minutes. The petri dishes were inverted and placed in transparent plastic bags tightly wrapped. The bags were then stored in the refrigerator overnight.

2.6 Disc Diffusion Assay for Microbial Sensitivity Testing

The dried crude extract was dissolved in their respective solvents to a stock solution of 20mg/m L. The stock solution was used to make a 10mg/m L solution. 12 ultraviolet sterilized paper discs of 6 mm (Whatman filter paper no. 1) in diameter were soaked in mini disposable petri dishes containing their respective extracts at the different concentrations for complete saturation. The agar plates were dried and sterilized using ultraviolet light in the laminar air flow cabinet.

The *C. albicans* which was prepared prior was dissolved in 10m L of distilled water in a test tube and its turbidity was compared to 0.5M Mc Farland Standard (1.5×10^8 - approximate bacterial suspension/ m L). Once identical turbidity is achieved, the mouth of the test tube is uncapped and flamed before a sterile cotton swab is submerged in the mixture.

The test tube is flamed and capped; the sterile cotton swab is streaked unto the agar plate in the different directions.

The soaked paper discs containing their respective plant extracts at different concentrations with metal hexacyanoferrate (II) suspension were transferred to the agar plate (four discs on one plate).

A positive control (concentrated Nystatin suspension) and negative controls (solvent only) were also used. The plates were taped to prevent any contamination when being transferred from the laminar air flow cabinet to the incubator. The plates were inverted and placed in an incubator at 37°C for 48 hours. The diameter zone of inhibition of the leaves extracts of *C. nucifera* and *A. esculentus* with metal hexacyanoferrates (II) suspension against *C. albicans* are given in Tables 3 and 4, respectively.

2.7 Phytochemical Screening

The phytochemical screening followed the methodology of Harborne [47] and Kokate [48]. The presence of alkaloids, tannins, saponins, flavonoids, volatile oils, phenols and steroids was determined for each leaves extract of *C. nucifera* and *A. esculentus*. The results are given in Tables 5 and 6.

Alkaloids: 2 m L of extract was measured in a test tube to which picric acid solution was added. An orange colour indicated the presence of alkaloids.

Tannins: A portion of the extract was diluted with water, 4 drops of 10% ferric chloride solution was added. A blue colour indicated gallitannins and green colour indicated catecholic tannins.

Saponins: About 0.5m L of extract was measured in a test tube and was shaken with 2 m L of water. If foam persisted for 10 minutes it indicated saponins.

Flavonoids: 4m L of extract solution was treated with 1.5 m L of 50% methanol solution. The solution was warmed and metal magnesium was added. To this solution, 6 drops of concentrated hydrochloric acid was added and red colour indicated flavonoids and orange colour indicated flavones.

Volatile Oils: 2m L of extract was shaken with 0.1m L dilute NaOH and a small quantity of dilute HCl. A white precipitate indicated volatile oils.

Phenols: 1m L of extract was treated with 4 drops of ferric chloride solution. A bluish black colour indicated phenols.

Steroids: 1m L of extract was treated with 0.5m L of acetic anhydride and 0.5 m L of chloroform. Then concentrated solution of sulphuric acid was slowly added. A greenish bluish colour indicated steroids.

2.8 Characterization of Metal Hexacyanoferrates (II) and Plant Extract

The spectral analysis of plant extract with each metal hexacyanoferrate (II) was recovered using Nujol Mull of **BUCK Scientific M530**.

All of the samples were placed in the infrared spectrophotometer and read at 4000 cm^{-1} - 400 cm^{-1} . A small drop of the plant extract with metal hexacyanoferrate (II) was placed on one side of the KBr window followed by one drop of Nujol Mull solution. The mixture was then covered and pressed with the other KBr window and placed in the IR spectrophotometer. These values are given in Tables 7 and 8.

2.9 Statistical Treatment

t- Test was used to compare the zones of inhibition of the plant extract only and plant extract with the addition of each metal hexacyanoferrate (II) at the same concentration. For each $p < 0.05$ - there is a significant difference, $p >$ there is no significant difference.

The inference “significant difference” implies that based on the zone of inhibition, the activities are not the same and thus the treatment is different. Therefore “no significant difference” would imply that the activities are the same, thus the treatments are the same.

Ultimately, it is to determine whether the addition of the metal hexacyanoferrate (II) is to be considered for its antifungal activity. Comparison of the zones of inhibition of the plant extract, with the addition of each metal hexacyanoferrate (II) at the different concentrations for those which are of opposite significant difference, will verify if concentration is a factor to be considered for its antifungal activity.

Comparisons were also conducted on the zones of inhibition of the plant extract with the addition of each metal (II) hexacyanoferrate, which demonstrated no significant difference in preliminary analysis and Nystatin suspension (positive control). Another preliminary analysis was conducted which compared the plant extract at the concentrations of the following to analyze if there was significant difference from Nystatin suspension.

Table 1. Element analysis of copper, manganese and nickel hexacyanoferrates (II)

Metal hexacyanoferrates (II)*	Percentage (%) found				
	Metal	Iron	Carbon	Hydrogen	Nitrogen
CuFc	37.3	15.4	14.7	3.1	18.1
MnFc	19.4	19.5	16.3	2.8	18.6
NiFc	20.8	19.6	20.3	1.5	21.4

*CuFc, copper hexacyanoferrate (II); MnFc, manganese hexacyanoferrate (II); NiFc, nickel hexacyanoferrate (II)

Table 2. Infrared spectral data of copper, manganese and nickel hexacyanoferrates (II)

Metal hexacyanoferrates (II)*	Adsorption frequency (cm^{-1})				
	H ₂ O molecules/OH groups	HOH bending	$\nu\text{C}\equiv\text{N}$ stretching	$\nu\text{Fe-C}$	Metal-N ^a
CuFc	3733	1628	2069	600	450
MnFc	3698	1632	2066	600	510
NiFc	3690	1619	2071	590	430

*CuFc, copper hexacyanoferrate (II); MnFc, manganese hexacyanoferrate (II); NiFc, nickel hexacyanoferrate (II)
^ametal-nitrogen band due to polymerization

3. RESULTS AND DISCUSSION

It can be observed from Tables 3 that the acetone extract of *C. nucifera* at 20 mg/m L and 10 mg/m L with manganese and copper hexacyanoferrates (II) demonstrated effective antifungal potential. The ethanol extracts at both concentrations showed no antifungal activity.

On the other hand in Table 4, all of the acetone extracts of *A. esculentus* at 20 mg/m L and 10 mg/m L indicated showed effective antifungal potential when paired with all three metal hexacyanoferrates (II) (with the exception of combining manganese hexacyanoferrate (II) with the plant extract at 10 mg/m L). The ethanol extracts at both concentrations demonstrated effective antifungal potential (with the exception of combining manganese hexacyanoferrate (II) with the plant extract).

The phenolic compounds such as phenols and flavonoids are antifungal agents. In Table 5, the phytochemical analysis of acetone and ethanol extracts of *C. nucifera* with copper, nickel and manganese hexacyanoferrate (II) suspensions.

The results revealed that the acetone extract combined with copper and manganese hexacyanoferrate (II) contained phenols.

Phenols were absent in the ethanol extract of *C. nucifera* with all three hexacyanoferrates (II).

Based on the data from Table 6, the phytochemical analysis of *A. esculentus* indicated that the acetone extracts when combined with copper, nickel and manganese hexacyanoferrates (II) contained phenols. While ethanol extracts when combined with nickel and manganese hexacyanoferrates (II) contained flavonoids and phenols, respectively.

A preliminary test was conducted on the plant extract only which was used for comparison. Based on the peaks before and after the addition of each metal hexacyanoferrate (II) to the plant extract, the disruption of the antifungal potential can be determined.

The infrared spectral data given in Table 7 showed that the antifungal potential was disrupted when the *C. nucifera*, ethanol extracts were combined with metal hexacyanoferrates (II). The exception was acetone extract with manganese and copper hexacyanoferrates (II).

The acetone and ethanol *A. esculentus* extracts combined with metal hexacyanoferrates (II) did not disrupt the antifungal potential when the infrared peaks were compared. The exception of the ethanol extract when combined with manganese hexacyanoferrate (II).

Interestingly the acetone extract when combined with manganese hexacyanoferrate (II) was partially disrupted based on the diminished sharpness of the peak. This is demonstrated in Table 8. The most effective antifungal activities were demonstrated by 10mg/m L ethanol extract of *A. esculentus* with nickel hexacyanoferrate (II) and 10mg/m L acetone extract of *A. esculentus* with copper hexacyanoferrate (II). This is shown in Figs. 1 and 2, respectively.

Table 3. Diameter zone of inhibition (mm) of extracts of *C. nucifera* leaves against *C. albicans*

Metal hexacyanoferrates (II) suspension* (10 mg/m L)	Acetone extract (mg/m L)		Ethanol extract (mg/m L)	
	20	10	20	10
NiFc	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
MnFc	7.6 ± 3.8	5.6 ± 4.5	0.0 ± 0.0	0.0 ± 0.0
CuFc	8.7 ± 1.1	8.7 ± 1.2	0.0 ± 0.0	0.0 ± 0.0

*CuFc, copper hexacyanoferrate (II); MnFc, manganese hexacyanoferrate (II); NiFc, nickel hexacyanoferrate (II)

Table 4. Diameter zone of inhibition (mm) of extracts of *A. esculentus* leaves against *C. albicans*

Metal hexacyanoferrate (II) suspension* (10 mg/m L)	Acetone extract (mg/m L)		Ethanol extract (mg/m L)	
	20	10	20	10
Nickel	5.1 ± 4.5	6.0 ± 4.5	4.8 ± 4.2	10.1 ± 6.5
Manganese	8.8 ± 0.6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Copper	10.5 ± 1.2	7.9 ± 3.8	8.9 ± 5.1	6.7 ± 4.1

*CuFc, copper hexacyanoferrate (II); MnFc, manganese hexacyanoferrate (II); NiFc, nickel hexacyanoferrate (II)

Table 5. Qualitative tests for the phytochemical analysis of acetone and ethanol extracts of

C. nucifera nickel, copper and manganese hexacyanoferrates (II) suspension

Phytochemical	Inference					
	Acetone extract			Ethanol extract		
	NiFc	CuFc	MnFc	NiFc	CuFc	MnFc
Alkaloids	-	-	-	+	-	+
Tannins	-	-	-	-	-	-
Saponins	+	+	+	+	-	+
Flavonoids	-	-	-	-	-	-
Volatile Oils	-	-	-	-	-	+
Phenols	-	+	+	-	-	-
Steroids	-	-	-	-	-	-

*CuFc, copper hexacyanoferrate (II); MnFc, manganese hexacyanoferrate (II); NiFc, nickel hexacyanoferrate (II).
(+) – present, (-) - not present

Table 6. Qualitative tests for the phytochemical analysis of acetone and ethanol extracts of A. esculentus nickel, copper and manganese hexacyanoferrates (II) suspension

Phytochemical	Inference					
	Acetone extract			Ethanol extract		
	NiFc	CuFc	MnFc	NiFc	CuFc	MnFc
Alkaloids	-	-	-	-	-	-
Tannins	-	-	-	-	-	+
Saponins	+	+	+	+	-	-
Flavonoids	-	-	-	+	-	-
Volatile Oils	+	-	+	-	-	-
Phenols	+	+	+	-	+	-
Steroids	-	-	+	+	+	+

*CuFc, copper hexacyanoferrate (II); MnFc, manganese hexacyanoferrate (II); NiFc, nickel hexacyanoferrate (II).
(+) – present, (-) - not present

Table 7. Infrared spectral data of acetone and ethanol extracts of C. nucifera and suspension of metal hexacyanoferrates (II)

Plant Extract [adsorption frequency (cm ⁻¹)]	Metal hexacyanoferrates (II) suspension*		
	NiFc	MnFc	CuFc
Acetone [below 600]	Antifungal potential not disrupted	Antifungal potential not disrupted	Antifungal potential not disrupted
Ethanol [3504, 3510]	Antifungal potential disrupted	Antifungal potential disrupted	Antifungal potential disrupted

*CuFc, copper hexacyanoferrate (II); MnFc, manganese hexacyanoferrate (II); NiFc, nickel hexacyanoferrate (II)

Table 8. Infrared spectral data of acetone and ethanol extracts of A.esculentusandsuspension of metal hexacyanoferrates (II)

Plant Extract [adsorption frequency (cm ⁻¹)]	Metal hexacyanoferrates (II) suspension*		
	NiFc	MnFc	CuFc
Acetone [2840]	Antifungal potential not disrupted	Antifungal potential partially disrupted	Antifungal potential not disrupted
Ethanol [below 600]	Antifungal potential not disrupted	Antifungal potential disrupted	Antifungal potential not disrupted

*CuFc, copper hexacyanoferrate (II); MnFc, manganese hexacyanoferrate (II); NiFc, nickel hexacyanoferrate (II)

To investigate the interaction of metal hexacyanoferrate (II) and natural antifungals, the samples were tested individually. Recent research revealed that *C. albicans* has developed the ability to utilize the

nitrogen present after the degradation of the cyanide bond to grow itself. This occurs due to the degradation of the metal-cyanide bond.



Fig. 1. 10mg/ml Ethanol extract of *A. esculentus* with nickel hexacyanoferrate (II)

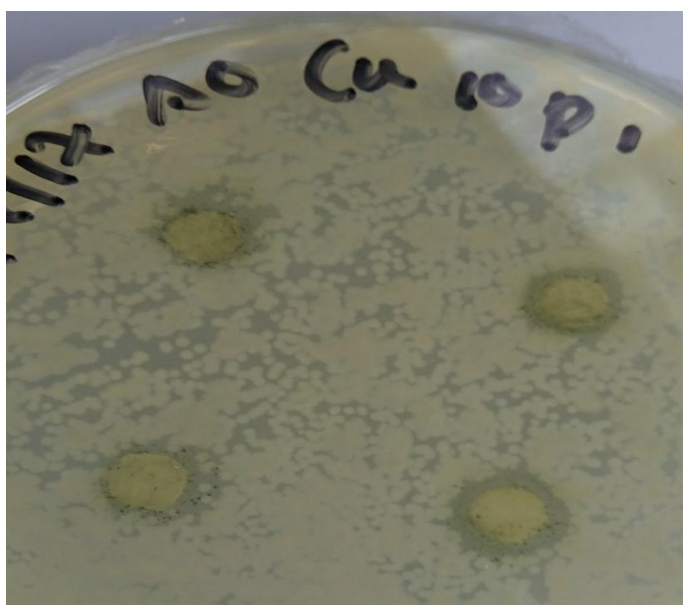


Fig. 2. 10mg/ml Acetone extract of *A. esculentus* with copper hexacyanoferrate (II)

The fungus also has the ability to use copper and manganese to neutralize the free radical attacks. The free radical attacks are necessary to fight against the fungus [49]. However, the acetone and ethanol extracts of *C. nucifera* and *A. esculentus* at the different concentrations exhibited antifungal potential.

When the acetone extract of *C. nucifera* interacted with manganese and copper hexacyanoferrate (II) at both concentrations, antifungal potential was exhibited.

While the acetone extract of *C. nucifera* with nickel hexacyanoferrate (II) and the ethanol extract with nickel, manganese and copper hexacyanoferrate (II) at both concentrations did not exhibit any inhibition [Table 3].

The interaction of the acetone and ethanol *A. esculentus* extract with nickel and copper hexacyanoferrate (II) at both concentrations, exhibited antifungal potential, while exhibiting antifungal potential was acetone extract of *A. esculentus* with manganese hexacyanoferrate (II) complex at 20 mg/m L. The interactions which showed antifungal potential indicated that the plant extract the metal hexacyanoferrate (II) had adsorbed the antifungal property from the natural antifungal. This is based on the observation that nickel and copper hexacyanoferrate (II) did not exhibit any antifungal potential at 10 mg/m L. The acetone extract of *A. esculentus* with manganese hexacyanoferrate (II) at 10 mg/ml and the ethanol extract with manganese hexacyanoferrate (II) at both concentrations did not exhibit any inhibition [Table 4].

The addition of the metal hexacyanoferrate (II) inhibited the natural antifungal's potential or the growth of the fungus fueled by the source of nitrogen was exceeded by the antifungal potential of the plant extract. A trend was observed with the interaction between the ethanol plant extracts and manganese hexacyanoferrate (II) at both concentrations, where there was no inhibition of the growth of *C. albicans*.

Ultimately, it was determined that the antifungal potentials of 10mg/m L of ethanol extract of *A. esculentus* and acetone extract with nickel and copper hexacyanoferrate (II) , respectively had no significant difference from that of Nystatin, a pharmaceutical treatment.

The presence of phenols has been observed in acetone extracts of *C. nucifera* with manganese and copper hexacyanoferrates (II). It was also present in acetone and ethanol extracts of *A. esculentus* with copper hexacyanoferrate (II). Mucilage from ochro pods have been tested for total phenolic content. A study which shows that eight mucilage samples gave an average of 37.42 mg GAE/g of total phenolics [49]. Phenolic compounds such as phenols and flavonoids (natural phenolic compound found in plants) have antifungal properties which enables their action against *C. albicans* and other *Candida* species [50].

Whereas the ethanol extracts of *A. esculentus* with nickel hexacyanoferrate (II) has been observed to contain flavonoids, a small quantity of phenol was observed for the acetone extract of *A. esculentus* with manganese hexacyanoferrate (II), which is a possible explanation as to why this extract at 20 mg/m L exhibited antifungal potential [Tables 5 and 6].

The acetone extracts of *C. nucifera* with nickel and copper hexacyanoferrates (II) showed similar IR spectra; however, their antifungal potential was different. This explanation also applies to ethanol extracts of *A. esculentus* with manganese and nickel hexacyanoferrates (II). All of the ethanol extracts of *C. nucifera* showed the absence of the peaks at 3504 cm^{-1} and 3510 cm^{-1} . The acetone extract of *A. esculentus* with manganese hexacyanoferrate (II) did not show as being a strong peak when compared to the plant extract. This could be the reason for its antifungal potential being exhibited at 20 mg/m L [Tables 7 and 8].

The antifungal potential of manganese, silver, titanium ferrocyanides, the extracts of *Azadirachta indica*, *Cassia alata*, *Cassia obtusifolia*, *Ocimum sanctum*, *Targetes patula* and their complexes against *A. niger* were studied by Tewari and Roberts [44]. The following order of antifungal activity was observed in metal ferrocyanides:

Titanium ferrocyanide (TiFc) > Silver ferrocyanide (AgFc) > Manganese ferrocyanide (MnFc).Titanium ferrocyanide and manganese ferrocyanide were found to have maximum and minimum antifungal property, respectively.

The observations of the bioassay test of metal ferrocyanides are given in Table 9.

The following order of antifungal potential was observed in 95 % ethanolic leaf extracts:

Azadirachta indica > *Tagetes patula* > *Ocimum sanctum* > *Cassia alata* > *Cassia obtusifolia*.

Azadirachta indica and *obtusifolia* were found to have maximum and minimum antifungal properties, respectively.

The observations of the bioassay test of natural antifungal extract with *A. niger* are given in Table 10.

The following order of antifungal activity was observed between natural antifungal with metal ferrocyanide complexes:

(i) Manganese ferrocyanide

Azadirachta indica > *Tagetes patula* > *Ocimum sanctum* > *Cassia alata* > *Cassia obtusifolia*

(ii) Silver ferrocyanide

Azadirachta indica > *Tagetes patula* > *Ocimum sanctum* > *Cassia alata* > *Cassia obtusifolia*

(iii) Titanium ferrocyanide

Azadirachta indica > *Tagetes patula* > *Ocimum sanctum* > *Cassia alata* > *Cassia obtusifolia*

Titanium ferrocyanide- *Azadirachta indica* and manganese ferrocyanide-*Cassia obtusifolia* complexes were found to have the maximum and minimum antifungal properties, respectively.

The observations of bioassay test of metal ferrocyanide-natural antifungal complexes with cultured fungal spore are given in Table 11.

It was also observed that fungal spores were able to grow in the control. It was unaffected by ethanol.

Table 9. Observation of bioassay test of metal ferrocyanides only with cultured fungal spores

MnFc	AgFc	TiFc
Evidence of few fungal spores growth was seen	Few spores of fungus were seen in area where silver ferrocyanide was not present	No evidence of fungal spores growth was seen
<i>Bioassay: 10 mg metal ferrocyanide per petri dish</i> <i>Room temperature: 30 ± 1°C; Time: 168 hrs.</i> <i>Cultured fungus: Aspergillus niger</i> <i>Order of antifungal activity: TiFc>AgFc>MnFc</i>		

Table 10. Observation of bioassay test of natural antifungals only with cultured fungal spores

<i>Azadirachta indica</i>	<i>Cassia alata</i>	<i>Cassia obtusifolia</i>	<i>Ocimum sanctum</i>	<i>Tagetes patula</i>
Evidence of small amount of spores was seen. It had the least evidence of fungal spores	Evidence of more fungal spores was seen but less than <i>Cassia obtusifolia</i> extract	Evidences of wide spread fungal spores was seen in comparison to all other plant extract	Evidences of fungal spores was seen but more than <i>Tagetes patula</i> extract	Evidences of small amount of spores was seen but more than <i>Azadirachta indica</i>
<i>Bioassay: 10 mg plant extract per petri dish</i> <i>Room temperature: 30 ± 1°C</i> <i>Time: 168 hrs.</i> <i>Cultured fungus: Aspergillus niger</i> <i>Order of antifungal activity: Azadirachta indica>Tagetes patula>Ocimum sanctum>Cassia alata>Cassia obtusifolia</i>				

Table 11. Observation of bioassay test of metal ferrocyanides and natural antifungals with cultured fungal spores

Metal ferrocyanide	<i>Azadirachta indica</i>	<i>Cassia alata</i>	<i>Cassia obtusifolia</i>	<i>Ocimum sanctum</i>	<i>Tagetes patula</i>
MnFc	Little sign of fungal growth seen	Some fungal spores seen growing but more than <i>O.sanctum</i>	Clumps of fungus were seen in the petri dish maximum growth	Few fungal spores seen growing more than <i>T.patula</i>	Evidence of small amount of fungal growth but more than <i>A.indica</i>
AgFc	Very little sign of fungal growth seen	More spores were seen growing than <i>O.sanctum</i>	Some spores were seen growing in clumps	Evidences of small amount of fungal growth but more than <i>T.patula</i>	Evidences of small amount of fungal growth but more than <i>A.indica</i>
TiFc	No evidence of fungal growth maximum inhibition	Evidences of small amount of fungal growth but more than <i>O.sanctum</i>	Evidences of small amount of fungal growth but more than <i>C.alata</i>	Few fungal spores seen growing but more than <i>T.patula</i>	Little fungal spores seen growing

Bioassay: 10 mg metal ferrocyanide and 10 mg antifungal plant extract per petri dish

Room temperature: 30 ± 1°C

Time: 168 hrs.

Cultured fungus: Aspergillus niger

4. CONCLUDING REMARKS

1. All of the ethanol extracts of *C. nucifera* did not exhibit antifungal potential with any of the metal hexacyanoferrates (II).
2. Ethanol and acetone extract of *A. esculentus* at both concentrations demonstrated antifungal potential when combined with copper and nickel hexacyanoferrates (II).
3. Acetone extracts of *A. esculentus* with manganese hexacyanoferrate (II) only showed antifungal potential at 20 mg/m L.
4. The metal hexacyanoferrate (II)-extracts of *A. esculentus* interaction will not demonstrate significant difference in the antifungal potential against *C. albicans*. This applies to 10 mg/m L of ethanol of *A. esculentus* with nickel hexacyanoferrate (II) and 10 mg/mL of acetone of *A. esculentus* copper hexacyanoferrate (II). Because there is no significant difference between the two latter interactions and Nystatin suspension, deduces that these possess the best antifungal potential.
5. It was observed that in some cases the metal hexacyanoferrates (II) did not inhibit the antifungal compounds: phenols and flavonoids.
6. The acetone extract of *C. nucifera* with copper and manganese hexacyanoferrates (II) contain phenols.
7. The acetone extract of *A. esculentus* combined with each of the metal hexacyanoferrates (II) contain phenols.
8. The ethanol extract of *A. esculentus* combined with copper hexacyanoferrate (II) contains phenol.
9. The ethanol extract of *A. esculentus* combined with nickel hexacyanoferrate (II) contains flavonoids.
10. Antifungal activity of some secondary metabolites are enhanced through interaction with metal ferrocyanides.
11. Titanium and manganese ferrocyanides were found to have maximum and minimum antifungal properties, respectively.

12. *Azadirachta indica* extract and *Cassia obtusifolia* were found to have maximum and minimum antifungal properties, respectively.
13. *Azadirachta indica* extract-titanium ferrocyanide complex and *Cassia obtusifolia* extract-manganese ferrocyanide complex were found to have the maximum and minimum antifungal properties, respectively.
14. It may also be concluded from this past study that titanium ferrocyanide-*Azadirachta indica* extract complex may be used as effective medicine for skin infection.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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